CFlow®

User Guide

Science is hard. Flow cytometry should be easy.®

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1 INTRODUCTION TO CFLOW

The Accuri CFlow® or CFlow Plus software (CFlow) allows you to control the C6 Flow Cytometer® system to acquire data, generate statistics, and analyze results. CFlow is both powerful and easy to use.

CFlow software provides the following features:

- Tabbed views for collection, analysis, and statistics
- Plots that display more than six decades of dynamic range
- Digital signal processing and compensation at any time
- Drag and drop plots
- Export files in FCS 3.0 format
- Seamless importation into FCS Express

1.1 Installing CFlow

To install CFlow:

1. Ensure the USB cable is *not* connected between the computer and cytometer.
2. Turn on the computer and monitor.
3. Insert the CFlow Software CD or flash drive provided by Accuri. When prompted, double-click on CFlowInstaller.exe.
4. Follow the instructions in the installation wizard.

1.2 Starting CFlow

Do not use CFlow until the C6 Cytometer has been completely set up (see the *C6 Flow Cytometer Instrument Manual*).

To open CFlow:

1. Double-click on the *CFlow* icon on the computer desktop. CFlow opens a new, blank workspace.

NOTE: If CFlow displays the message *Extra startup time needed due to cleaning or improper shutdown,* the C6 will take several more minutes than usual to recover and return to the green-light ready state. This may occur on initial C6 startup after you receive the unit. It will also occur after an interruption of power to the unit.

1.3 CFlow Workspace

The main CFlow window is called the CFlow workspace. The workspace contains controls and displays that provide access to all the functions for acquiring and analyzing data. The CFlow workspace is organized on three separate tabs:

- *Collect*—Contains controls for setting up data collection and acquiring data (see chapter 3 for details).
- *Analyze*—Contains controls for analyzing data (see chapter 4 for details).
- *Statistics*—Displays statistical information (see chapter 5 for details).

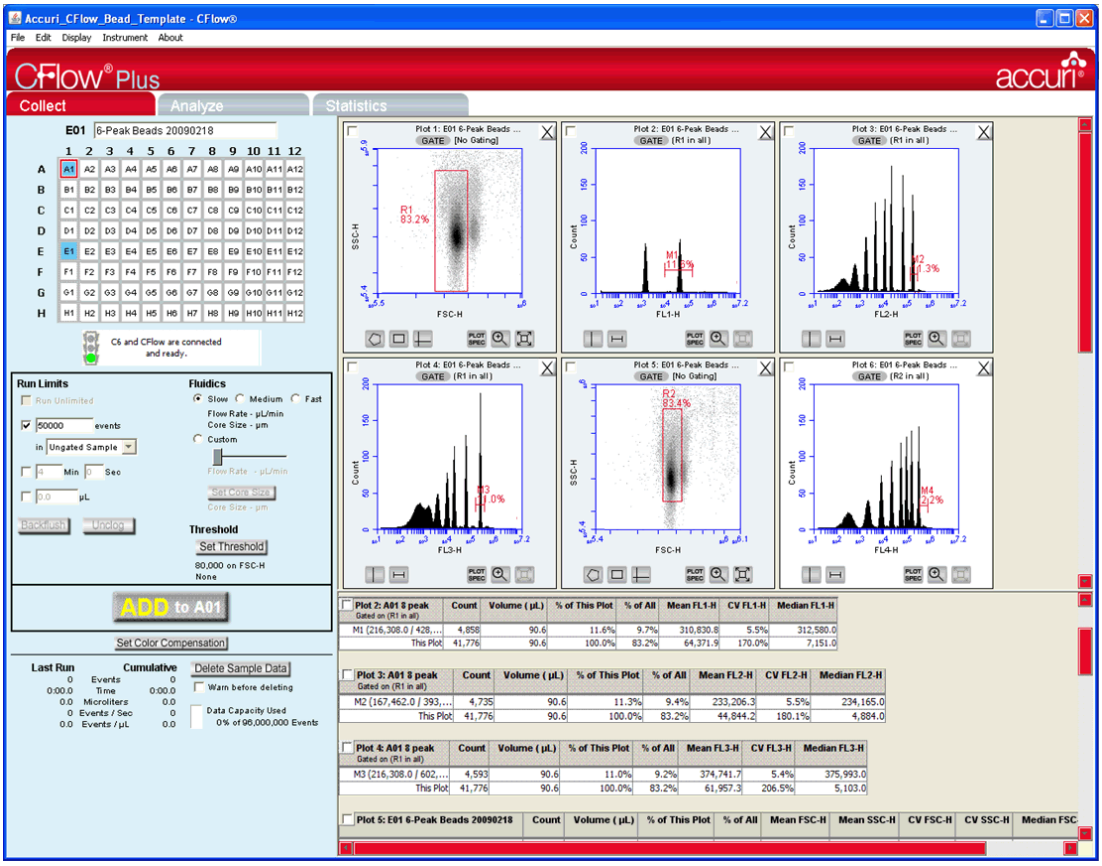


Figure 1-1. *Collect* Tab Workspace

1.4 Opening a New CFlow Workspace

When CFlow opens, it displays a blank workspace with no custom settings. You can use a new CFlow workspace for creating a new analysis template.

To open a new workspace:

1. Do one of the following:
   - If CFlow is not already open, double-click on the CFlow icon on the computer desktop.
   - If CFlow is already open, select *File* > *New CFlow File*. CFlow prompts you to save any unsaved changes to the previous workspace.

1.5 Exiting CFlow

To exit CFlow:

1. Select *File* > *Quit.*
2. If prompted to save changes to the CFlow workspace, do one of the following:
   - Click on the *Yes* button to save changes.
   - Click on the *No* button to close CFlow without saving changes.
   - Click on the *Cancel* button to cancel the exit and keep CFlow open.

1.6 Using the Example CFlow File

An example CFlow file of a four-color analysis of human peripheral blood (HPB 4 Color Tutorial.c6) can be downloaded from the Accuri website (www.accuricytometers.com/technical_information/tutorials/). You can use the data in this file to explore various tools in CFlow without the worry of corrupting or losing your own experimental data. Figures throughout this user guide show data from the example file.

To create the example file, four sample tubes were used to assess the $CD3^+CD4^+$ and $CD3^+CD8^+$ cell populations. These samples were prepared by staining peripheral blood with directly conjugated antibodies, followed by red cell lysis, according to standard methods.

The following table describes the experimental design:

- Tube 1—Background control
- Tube 2—White blood cell gating control
- Tube 3—Control required for determining the percent of $CD4^+$ and $CD8^+$ cells within the $CD45^+CD3^+$ subset
- Tube 4—Actual test sample

Table 1-1. Experimental Design for HPB 4 Color Tutorial File

Fluorochrome	*Sample Tube*			
	Tube 1	Tube 2	Tube 3	Tube 4
FITC	Isotype	Isotype	**CD3**	**CD3**
PE	Isotype	Isotype	Isotype	**CD4**
PE-Cy7	Isotype	**CD45**	**CD45**	**CD45**
APC	Isotype	Isotype	Isotype	**CD8**

This experimental design does not contain single-stained fluorescence controls. The only channels where fluorescence spillover causes ambiguity for setting gates are FITC spillover into PE and PE-Cy7 spillover into the FITC and PE channels. For details on correcting fluorescence spillover, see section 3.12.

2 VALIDATING THE PERFORMANCE OF THE C6

Perform a validation of the fluidics at least once each day that you use the C6. This ensures that the C6 is working properly before running experimental samples.

Use the same CFlow file each day to collect validation bead data so that you can compare trends over time. When you run a validation, advance to the next empty well in row A-D (for 8-peak beads) or E-H (for 6-peak beads). Include the date in the *Sample Naming Field* when you name the sample to keep track of each day's validation. Start a new validation bead file when you fill up the wells.

Reagents required:

- Spherotech 8-Peak Validation Beads (Accuri Part# QA-100, supplied with the initial C6 shipment)
- Spherotech 6-Peak Validation Beads (Accuri Part# QA-110, supplied with the initial C6 shipment)
- Sheath fluid: De-ionized, filtered water (0.2 µm filter) plus Bacteriostatic Concentration Solution (Accuri Part# KR-220, supplied with the initial C6 shipment)

2.1 Running Validation Beads

To run the validation beads:

Setup:

1. If this is the first time using the C6 Cytometer, open a CFlow file and run filtered de-ionized water from a 12x75 mm sample tube for at least 15 minutes.
2. Verify that the file named CFlow 8 & 6 Peak Bead Template.c6t has been copied to the CFlow computer. The file is located on the CFlow CD or flash drive and is on the Accuri website (www.accuricytometers.com/technical_information/templates/).
3. Select *File > Open CFlow File or Template.*
4. In the Open dialog box, browse to the location of the template file and open the file.

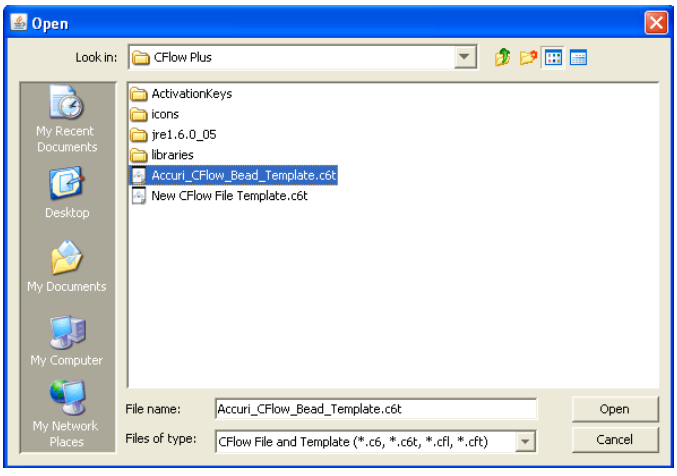


Figure 2-1. Open the Bead Template

Clean the SIP:

5. Click on the first empty well in rows A-D to advance to the well.

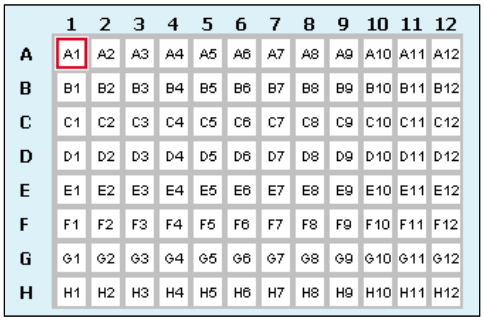


Figure 2-2. Select an Empty Well

6. Place an empty 12x75 mm tube on the SIP.
7. Click on the *Backflush* button.
8. After the backflush, place a fresh tube with 2 mL of filtered, de-ionized water on the SIP.
9. Disable the *Run Unlimited* check box in the Instrument Control Panel.

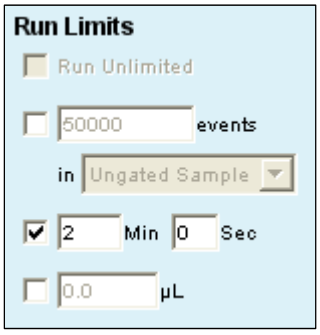


Figure 2-3. Run Limits: Disable Run Unlimited

10. Enable the *Time* check box next to the *Min* and *Sec* fields in the Instrument Control Panel and type in a run time of two minutes.
11. Select the *Fast* radio button in the Fluidics section of the Control Panel.
12. Click on the *RUN* button to rinse out the SIP.
13. Once the run is finished, click on the *Delete Sample Data* button to delete data collected during the rinse.
14. Remove the tube from the SIP.

Run 8-Peak Validation Beads:

15. Disable the *Time* check box next to the *Min* and *Sec* fields and enable the *Events* check box to stop the run when the specified number of events has been acquired.

16. Type *50000* in the *events* edit box and select *Ungated Sample* from the associated drop-down list.

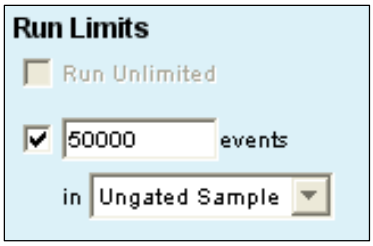


Figure 2-4. Run Limits: 50000 Events

17. Vortex a sample tube containing suspended 8-peak validation beads, prepared according to the package instructions. Place the tube on the SIP.
18. Select the *Slow* radio button in the Fluidics section of the Control Panel.
19. Click on the *RUN* button to start acquisition. Acquisition automatically stops after 50,000 total events are acquired.

    CAUTION: Make sure the well in CFlow is empty before starting the run. If the button displays *ADD TO,* the well already contains data.

    NOTE: The R1 region may not encompass the main population of bead events on the FSC-H vs. SSC-H plot. This is common and acceptable at this stage.
20. Name the sample by typing a name in the text box just above the Sample Grid. Include the date in the sample name to differentiate it from samples collected on other dates.

    NOTE: You can also name samples before, during, or after collection.

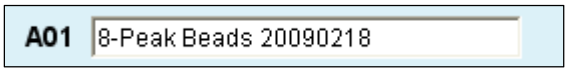


Figure 2-5. Sample Name: 8-Peak Beads

21. When the collection is finished, remove the sample tube and wipe off the end of the SIP with a lint-free tissue (or similar material) to minimize sample carryover.

Run 6-Peak Validation Beads:

22. Vortex a tube of suspended 6-peak validation beads, prepared according to the package instructions. Place the tube on the SIP.

23. Click on the first empty well in rows E-H to advance to the well.

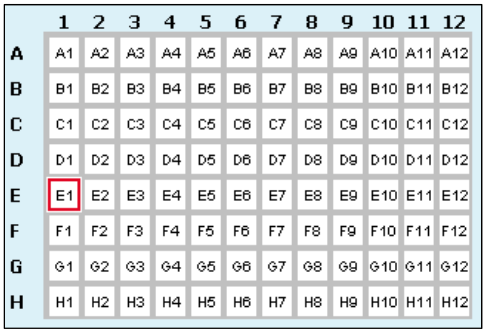


Figure 2-6. Select an Empty Well

24. Verify that *Events* is still enabled and set at 50,000 in *Ungated Sample.*

Figure 2-7. Run Limits: 50000 Events

25. Click on the *RUN* button.

    NOTE: The R2 region may not encompass the main population of bead events on the FSC-H vs. SSC-H plot. This is common and acceptable at this stage.

26. Name the sample with a name that includes the date processed.

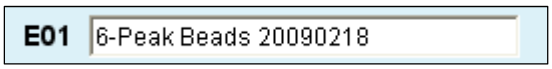


Figure 2-8. Sample Name: 6-Peak Beads

End The Procedure:

27. When the collection is finished, remove the sample tube from the SIP and wipe off the end of the SIP with a lint-free tissue.
28. Place a tube with 2 mL of filtered, de-ionized water on the SIP and advance to any empty data well.

29. Select the *Time* check box (*Min Sec*) in the Instrument Control Panel and set it for two minutes.

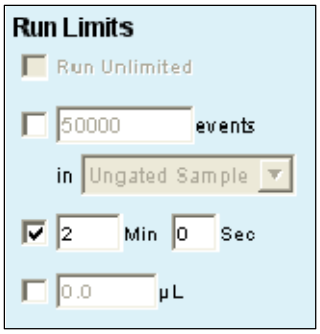


Figure 2-9. Run Limits: 2 Minutes

30. Click on the *RUN* button.
31. When the run is finished, leave the tube on the SIP.

2.2 Saving Validation Bead Data

By default, CFlow automatically saves validation bead data at the end of each sample. You can also save data manually at any time. For information on saving data, see section 3.10.

2.3 Analyzing and Recording Validation Bead Data

After you collect the bead data, analyze the data using the *Collect* tab of CFlow to ensure that the C6 is functioning properly.

To analyze the bead data:

1. Click on the well that contains the most recent 8-peak bead data (in rows A-D).
2. On the first FSC-H vs. SSC-H plot (scatter plot) in the bead file, adjust the pre-drawn region (R1) to encompass the main population by dragging the border of the region (see Figure 2-10). R1 should contain 75-85% of all events.

NOTE: There is usually a “shadow” population (called *bead doublets* or *clumps*) that is slightly higher in FSC-H than the main cluster of beads; this is normal for these beads. Do not include the shadow group in R1.

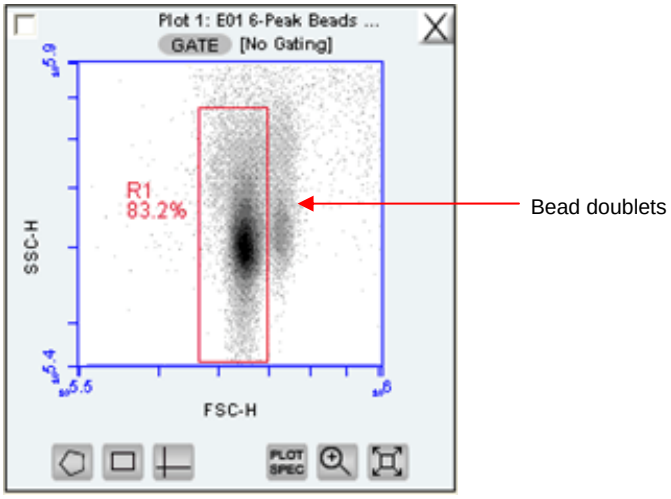


Figure 2-10. Plot with Bead Doublets

3. Verify that the next three plots (FL1-H, FL2-H, and FL3-H) are gated on scatter region R1 and that the plots display the message *R1 in all* next to the *GATE* button (Figure 2-11). If it is not displayed, click on the *GATE* button and select *R1 on all events* from the pop-up dialog box. For information about gates, see section 3.6.

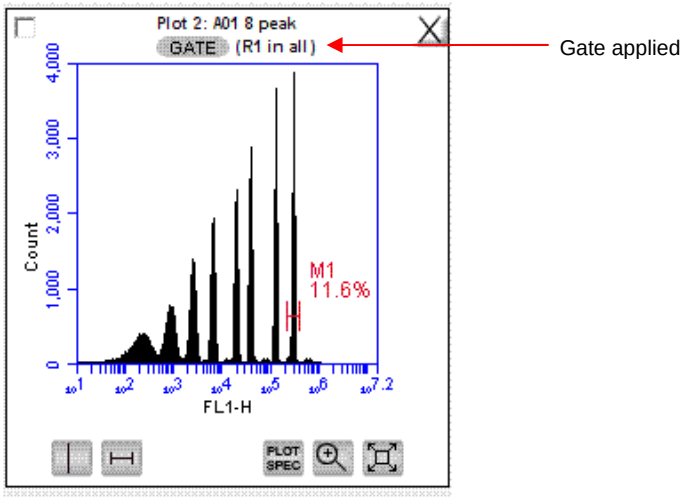


Figure 2-11. Gate Applied to 8-Peak Bead Plot

4. Measure the CV of the top (brightest, far right) peak on each of the three fluorescence plots. To place the predrawn horizontal marker tightly around the peaks:
   - Use the Zoom Tool in the plot to zoom in on the top peak (see section 3.9).
   - Adjust the marker tightly around the peak by clicking on the marker and dragging its edges.
   - Click on the Expand Tool to zoom back out.

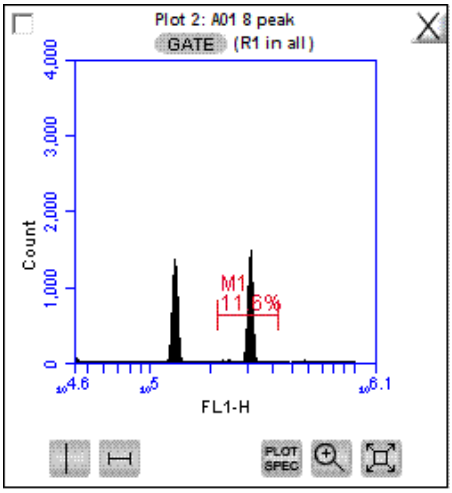


Figure 2-12. Zoomed View of Plot

5. Compare the bead run to the manufacturing results sent with the C6. If the C6 is performing properly, the data plots should look similar to the 8-peak bead plots in Figure 2-13. Look for the following:
   - One main population of beads on FSC-H vs. SSC-H (a shadow population is acceptable)
   - Eight discernable peaks on FL1-H and FL2-H
   - At least six peaks on FL3-H

   NOTE: FL4-H performance is checked with the 6-peak beads in steps 6-9.

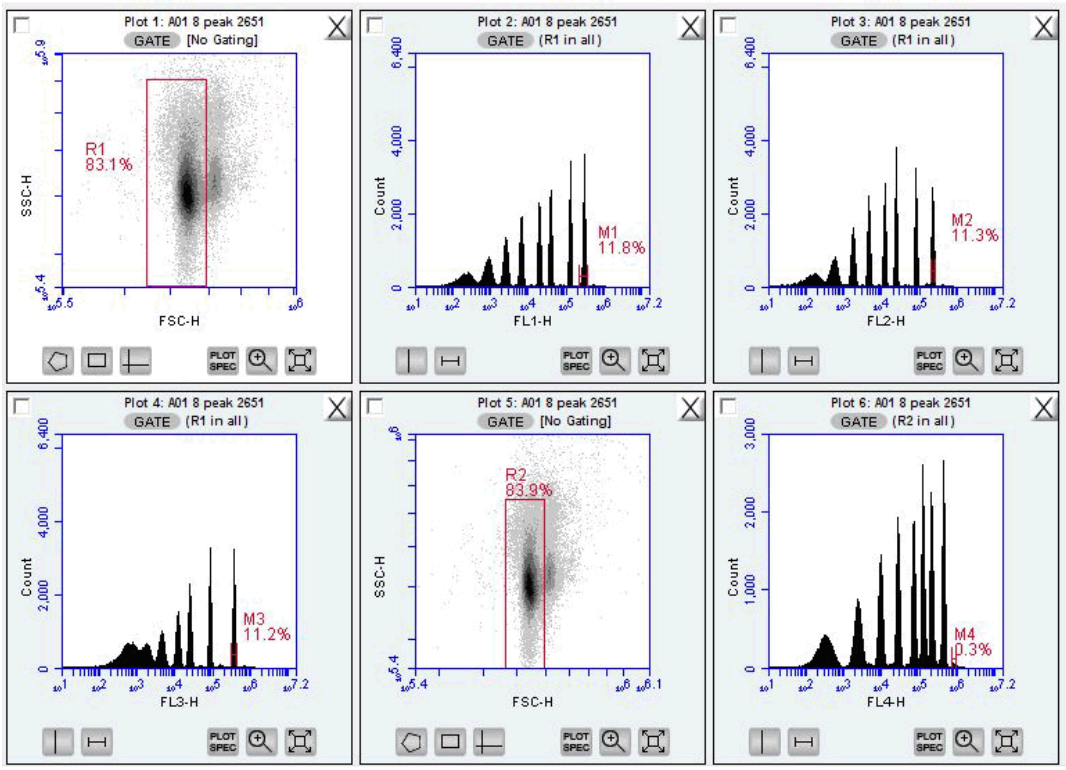


Figure 2-13. 8-Peak Validation Bead Data

6. Select the well containing the most recent 6-peak bead data (in rows E-H).

7. Adjust the pre-drawn region R2 in the 6-peak scatter plot FSC-H vs. SSC-H to encompass the main population (similar to the procedure for the 8-peak beads). This population should look like an exclamation point. The R2 region should encompass the entire exclamation point (Figure 2-14).

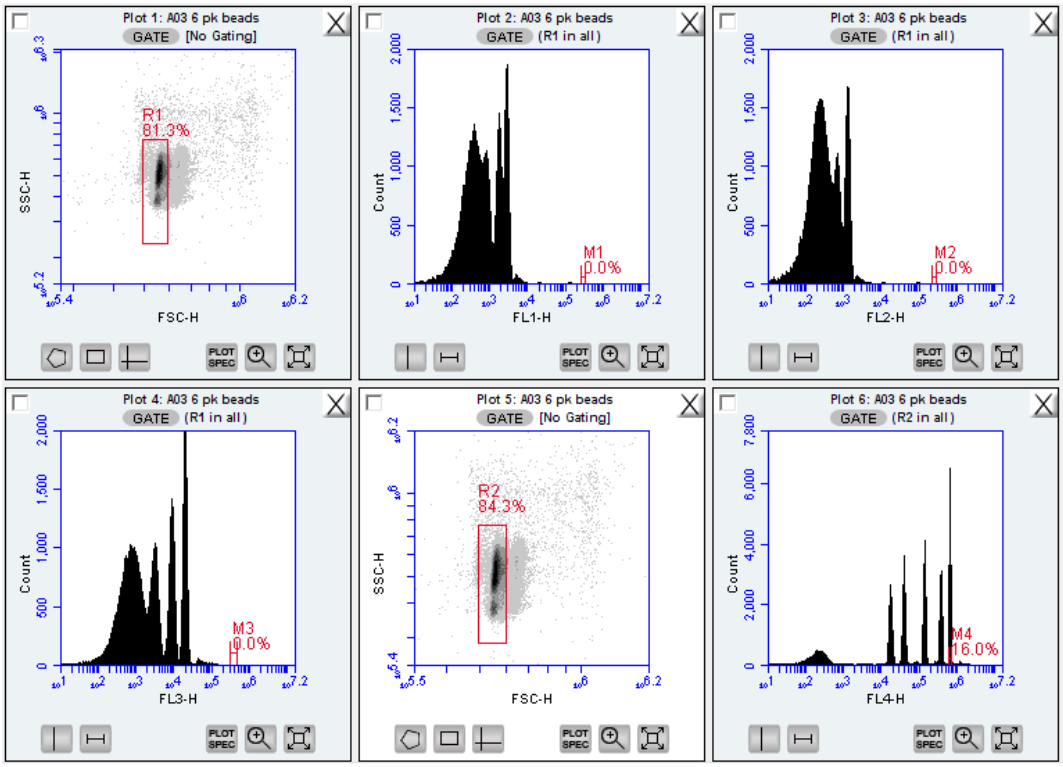


Figure 2-14. 6-Peak Validation Bead Data

8. Verify that the FL4-H plot is gated on region R2. If it is not, click on the *GATE* button and select *R2 on all events* from the pop-up dialog box.
9. Measure the CV of the top (brightest, far right) peak by adjusting the marker in the FL4-H plot so that it is placed tightly around the peak. See the plots in Figure 2-14 for an example of good 6-peak bead data. Look for the following:
   - One main population of beads on FSC-H vs. SSC-H
   - Six peaks on FL4-H
10. If you want, you can record the following information for each parameter in the Accuri C6 Cytometer Log (available on the CFlow CD or flash drive):
    - Number of peaks
    - Mean channel numbers for the top peaks and forward scatter
    - CVs for the top peaks and forward scatter
11. If this is your first validation run, send a copy of the file to Accuri Technical Support: techsupport@accuricytometers.com (USA) or eurotech@accuricytometers.com (Europe) for analysis.

2.4 Monitoring Validation Bead Data

You can easily monitor validation bead data (and thus the C6 performance) over time in one CFlow file to easily determine if the C6 performance is stable.

To monitor the bead data:

1. Save the 8- and 6-peak validation bead data from each day in separate wells.
2. Create a table in the *Statistics* tab that contains the mean channel numbers and CVs for the top peaks and forward scatters (see chapter 5 for details on creating tables in the *Statistics* tab). The C6 reports arithmetic means.

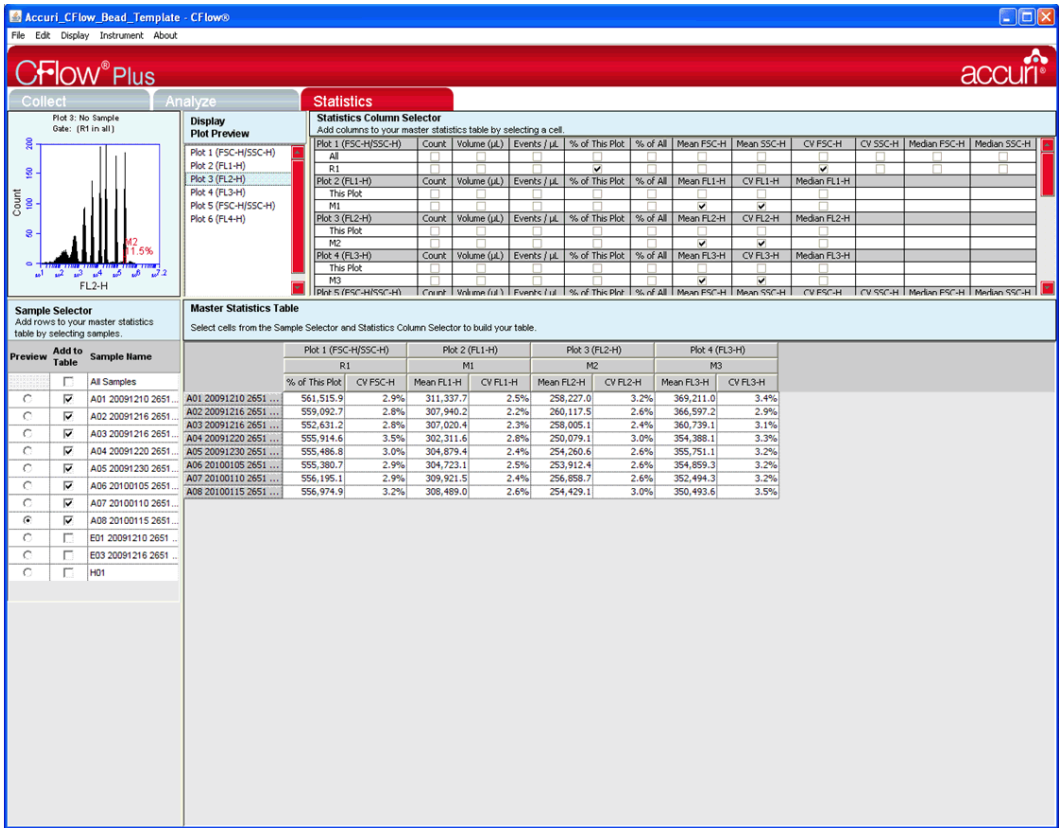

Accuri_CFlow_Bead_Template - CFlow®
File Edit Display Instrument About
CFlow® Plus
accuri®
Collect
Analyze
Statistics
Statistics Column Selector
Add columns to your master statistics table by selecting a cell.
Display
Plot Preview
Sample Selector
Add rows to your master statistics table by selecting samples.
Master Statistics Table
Select cells from the Sample Selector and Statistics Column Selector to build your table.

Figure 2-15. *Statistics* Tab: 8-Peak Bead Data from Successive Days

3. Compare statistics over time for trends or sudden changes in mean values to validate the cytometer's performance.

2.5 Troubleshooting Validation

The following conditions may indicate a problem with the C6 or the validation beads:

- Very broad CV (> 5.0%) or multiple populations for FSC-H on the 8- or 6-peak beads (excluding the doublet population).
- Fewer than eight peaks for FL1 or FL2.
- Fewer than six peaks for FL3.
- Fewer than six peaks for FL4.
- The brightest peak CVs are > 5.0%.

To troubleshoot validation:

1. If bead data were not acquired using the slow rate, select *Slow*, resuspend the beads, and recollect the data.
2. If the beads have been diluted for more than one week, kept at room temperature or warmer, or exposed to light for long periods of time, their performance may be substandard. Make new bead suspensions and run the bead sample again.
3. There may be a bubble or clog in the flow cell. Do one or more of the following:
   - Run the bead sample again.
   - Remove the tube from the SIP, place an empty tube under the SIP, and click on the *Unclog* button. When the unclog cycle is finished (CFlow displays a green Traffic Light), run the bead sample again.
   - Remove the tube from the SIP, place an empty tube under the SIP, and click on the *Backflush* button. When the cycle is finished (CFlow displays a green Traffic Light), run the bead sample again.

3 COLLECTING DATA

The *Collect* tab allows you to set data collection criteria, start and stop data acquisition, and view data on collected samples. The tab contains buttons and controls for performing the following functions:

- Controlling sample collection
- Acquiring data
- Creating plots (histogram, dot, or density) for viewing data
- Setting stop criteria and thresholds
- Controlling the fluidics
- Using regions and markers to create gates and obtain statistics
- Saving and printing plots and data
- Accessing the following analytic functions in CFlow:
  - Creating complex gates
  - Setting fluorescence compensation
  - Generating statistics

3.1 Viewing the *Collect* Tab

CFlow displays the *Collect* tab when CFlow opens. You can also view the tab by clicking on *Collect* from the *Analyze* or *Statistics* tabs.

The *Collect* tab is organized into two major sections:

- Instrument Control Panel—Panel on the left side of the window that contains controls for collecting data.
- Data display—Large area on the right side of the window that shows sample data in plots and in a Statistics Table.

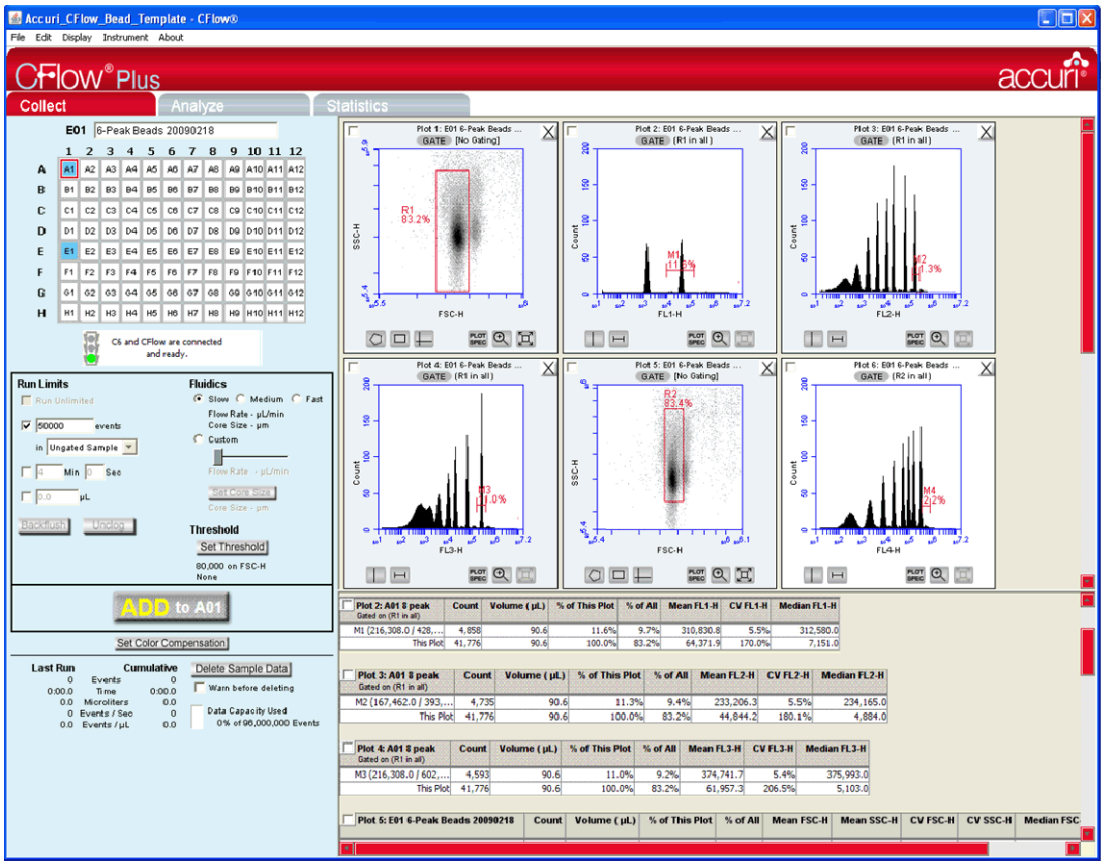


Figure 3-1. *Collect* Tab Workspace

The following table describes each of the controls and indicators in the *Collect* tab:

Table 3-1. *Collect* Tab Controls

Control	*Description*
Sample Naming Field	Text box for naming the sample that you are collecting.
Sample Grid	Matrix laid out in the configuration of a 96-well plate to help organize experiments and collect data from sample tubes. CFlow acquires each sample into its own well in the Sample Grid. The wells can be filled with data in any order. The wells are color-coded: • White—Does not contain data. • Blue—Contains data. • Red outline—Currently selected for viewing or collecting data.

Control	*Description*
Traffic Light and Message	Indicator that displays CFlow's readiness and system messages. Before data collection can begin, CFlow must display a green Traffic Light with the message *C6 and CFlow are connected and ready.* The Traffic Light status is color-coded: • Green—CFlow is ready to collect data or is collecting data. • Yellow—The C6 is preparing to perform an action or a non-critical error has occurred. • Red—A critical error has occurred.
Run Limits	Contains a set of controls that allow you to define criteria for automatically stopping data collection. See section 3.2.3 for details.
Fluidics	Contains a set of controls for defining flow rate and core size. See section 3.2.1 for details.
Backflush	Performs a backflush to eject sample from the SIP. See section 6.1 for details.
Unclog	Performs an unclog cycle to purge the flow cell of debris. See section 6.2.1 for details.
Threshold	Sets the event threshold to gate out debris and noise from cell samples. The default value is 80,000 on FSC-H. See section 3.2.2 for details on setting threshold values.
RUN/PAUSE/ADD TO	Toggle button that performs the following functions: • *RUN*—Starts the sample acquisition. • *PAUSE*—Pauses the acquisition. Click on *ADD TO* to resume data collection. • *ADD TO*—Allows you to collect more sample in a well that already contains data.
Set Color Compensation	Opens the Color Compensation dialog box for correcting fluorescence spillover. See section 3.12 for details.
Acquisition Counters	Displays the following information about the most recent acquisition for the selected well *(Last Run)* and all acquisitions for the selected well *(Cumulative)* in real-time: • *Events*—Number of events sampled. • *Time*—Elapsed acquisition time. • *Microliters*—Volume of acquired sample. • *Events/sec*—Events acquired per second. When the run is completed, this is the average value. • *Events/µL*—Events acquired per microliter. When the run is completed, this is the average value.
Delete Sample Data	Permanently deletes all events from the current sample. Enable the *Warn before deleting* check box to display a warning message before deleting sample data. Also contains a *Data Capacity Used* meter that displays the amount of data storage capacity currently used in CFlow.

Control	*Description*
Plots Pane	Area displaying two rows of plot corrals for graphically viewing data on the selected sample. Scroll up or down to view additional plots. You can create multiple plots for each sample. Each plot corral contains buttons for creating histogram, scatter, and dot plots. For information on creating and using plots, see section 3.4.
Statistics Table	Table below the plots that displays statistical information on individual plots.

3.2 Collecting Sample Data

Each data well holds a maximum of 1 million events. You can add more events (up to a total of 1 million) to a well at any time, even if the well already contains data. When a data well already contains data, the *RUN* button displays *ADD TO*.

CFlow must display a green Traffic Light and the message *C6 and CFlow are connected and ready* to collect data.

Figure 3-2 below shows a new workspace. Only a density plot of linear FSC-A vs. linear SSC-A is displayed. The plot is already zoomed to show channels 0 to 1,600,000 on FSC-A and 0 to 800,000 on SSC-A.

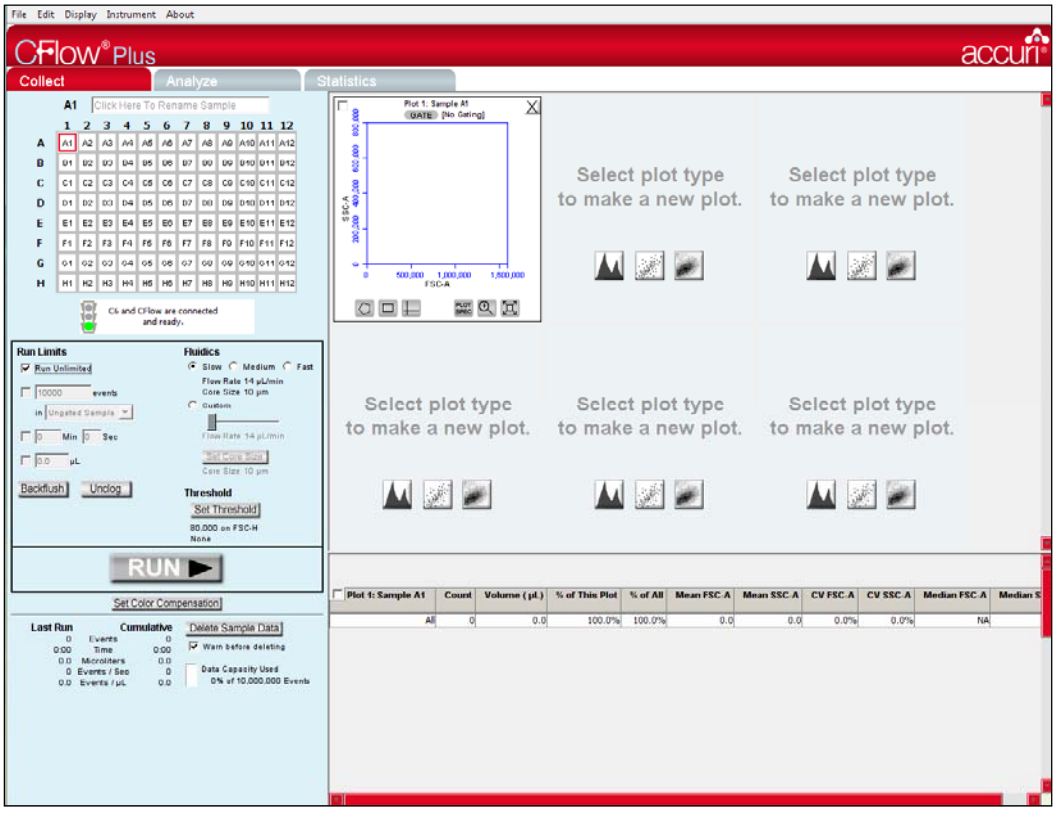


Figure 3-2. New CFlow Workspace

To collect sample data:

1. Set the fluidics rate (see section 3.2.1), threshold (see section 3.2.2), and run limits (see section 3.2.3).
2. Optionally, name the sample (see section 3.2.4).
3. Collect sample data (see section 3.2.5).
4. Open plots (see section 3.4).
5. Set and apply gates (see section 3.6).
6. Name plot axes (see section 3.8).
7. Apply fluorescence compensation values (see section 3.12).
8. Analyze the sample data (see chapter 4).

Alternatively, you can collect all data with minimal setup and perform analysis at a later time (including setting gates and applying fluorescence compensation).

To collect data with minimal setup:

1. Set the run limits, fluidics rate, and thresholds.
2. Collect the data for all samples.

3.2.1 Setting the Fluidics Rate

The system can accommodate an upper limit of 10,000 events per second, but it is recommended to acquire samples at a rate of 2,500 events per second or less to ensure the best data resolution.

To set the fluidics rate:

1. Click on the *Slow, Medium,* or *Fast* radio button in the *Fluidics* section of the *Collect* tab.

   NOTE: It is recommended to start data collection on slow and observe the data rate. You can then adjust the setting to medium or fast, if necessary.

You can customize your fluidics rate settings and adjust the sample core size for a range of cell sizes.

3.2.2 Setting the Threshold

Thresholds gate out debris and noise from cell samples so that sample data are not compromised. By default, CFlow filters out events lower than channel 80,000.

You can change the threshold settings at any time before, during, or after data acquisition. The primary threshold is the parameter that triggers data collection. You can optionally set a secondary threshold to filter out additional data. FSC-H is the default primary threshold.

CAUTION: Take care when setting thresholds before or during data collection. Any event not meeting the threshold criteria will be not be acquired or saved. When changes are made to the threshold values after data collection, CFlow displays a warning message if the new threshold value will result in permanent data loss.

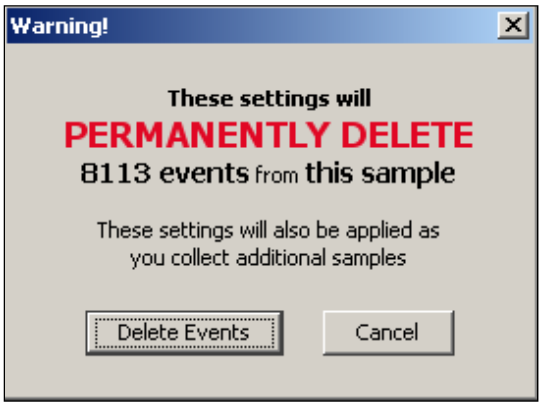


Figure 3-3. Threshold Settings Warning Message

To set the threshold:

1. Do one of the following:
   - Select *Instrument > Set threshold.*
   - Click on the *Set Threshold* button in the Instrument Control Panel.
2. Select the primary threshold parameter from the *Primary Threshold* drop-down list in the Threshold Settings dialog box.

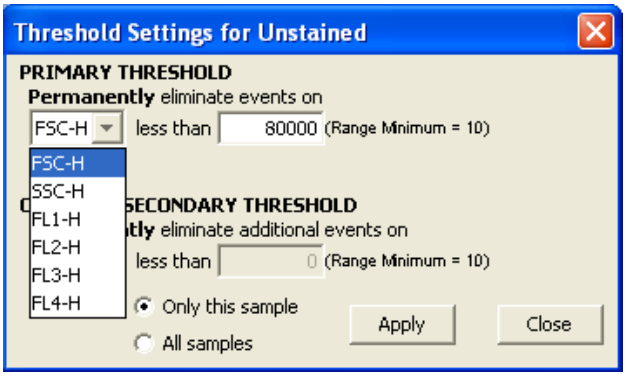


Figure 3-4. Primary Threshold Drop-Down List

3. Type *80000* in the *less than* edit box to set the threshold minimum to channel 80,000.

   NOTE: You may need to set a lower or higher FSC-H threshold when working with small cells (such as platelets or bacteria) or large cells (such as cell lines), respectively.
4. If you want to apply a secondary threshold for filtering out more data, do the following:
   - Select the threshold parameter from the *Secondary Threshold* drop-down list.
   - Type a value in the *less than* edit box to set the threshold minimum.

5. Do one of the following:
   - Select the *Apply to All samples* radio button to apply settings to all samples, including all previously collected data in other data wells.
   - Select the *Apply to Only this sample* radio button to apply settings to the current sample only.

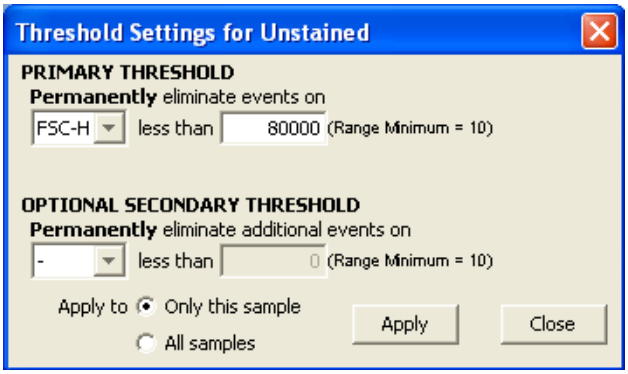


Figure 3-5. Threshold Settings Dialog Box

6. Click on the *Apply* button to apply the threshold settings.
7. Click on the *Close* button to close the dialog box.

3.2.3 Setting a Run Limit

Set a run limit to indicate to CFlow when to stop collecting data. You can set a run limit based on any of the following criteria:

- After a specified time
- After a specified volume
- After a specified number of events
- For an unlimited time (until you manually stop the run)

You can also choose multiple run limits (the C6 stops on the first limit reached).

For the first sample of your experiment, it is recommended to collect a total of 100,000 to 200,000 ungated events. You can change the run limit once a gate has been set on the population of interest.

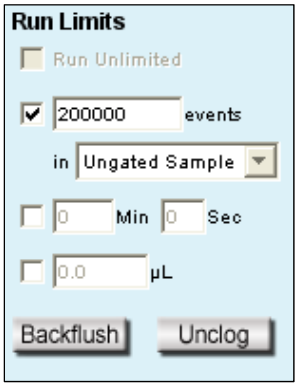


Figure 3-6. Run Limits Controls

To collect samples without setting a run limit:

1. Disable all check boxes in the Run Limits section of the *Collect* tab.
2. Enable the *Run Unlimited* check box.

To stop the run after a specified number of events have been sampled:

1. Enable the check box next to the *events* field.
2. In the associated text box, type the number of events at which to stop the run.
3. Do one of the following in the drop-down list below the text box:
   - Select *Ungated Sample*.
   - Select a gating strategy (if one exists) to stop the run when the assigned number of events has been collected in the gating region.

To stop the run after a time has expired:

1. Enable the check box next to the *min* and *sec* fields.
2. Type the number of minutes *(min)* and seconds *(sec)* at which to stop the run.

To stop the run after a specified volume has been sampled:

1. Enable the check box next to the *µL* field.
2. Type the volume in microliters (µL) at which to stop the run.

3.2.4 Naming the Sample

You can name samples at any time. If you do not type in a name, CFlow names the sample according to the well location (for example, `A01`).

To name the sample:

1. Type the sample name into the text box above the 96-well grid.

Figure 3-7. Sample Name Field

3.2.5 Running the Sample

To run the sample:

1. Gently resuspend the cells in the sample tube and place the tube on the SIP.
2. Select an empty sample well in CFlow.
3. Click on the *RUN* button to start the sample collection.

CFlow begins fluidics initialization. During this time the Traffic Light turns yellow and CFlow displays the message *Preparing to analyze sample*. Once initialization is complete, the Traffic Light turns green and CFlow displays the message *C6 is collecting data*. The current well flashes blue during data collection. After the run limit is reached, the well stops flashing and remains blue, indicating that the well contains data.

4. If desired, you can collect more data in a well that contains data by clicking on the *ADD TO* button at any time.

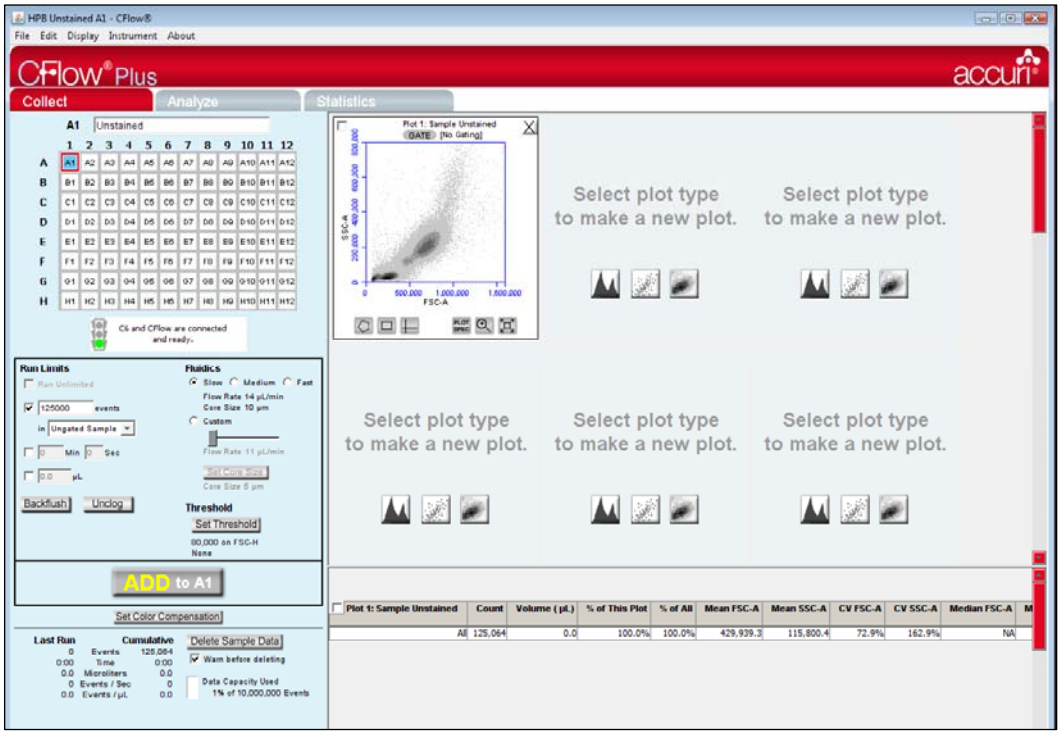


Figure 3-8. CFlow Workspace after Collecting a Sample

3.2.6 Adding New Sample Data to a CFlow File

You can collect new samples and add the data to a CFlow file that already contains sample data, or you can add new data to an empty sample well.

To add data to a CFlow file:

1. Remove the previous sample tube from the SIP and wipe the outside of the SIP with a lab wipe to minimize carryover between samples.
2. Gently resuspend the new sample and place the tube on the SIP.

   NOTE: You do not need to perform a backflush between samples. Wipe the SIP with a lab wipe to prevent carryover.

3. Click on a data well in the 96-well sample grid. If you select an empty well, any plots and gates you created earlier are still displayed, but they do not contain any data, as shown in the following figure.

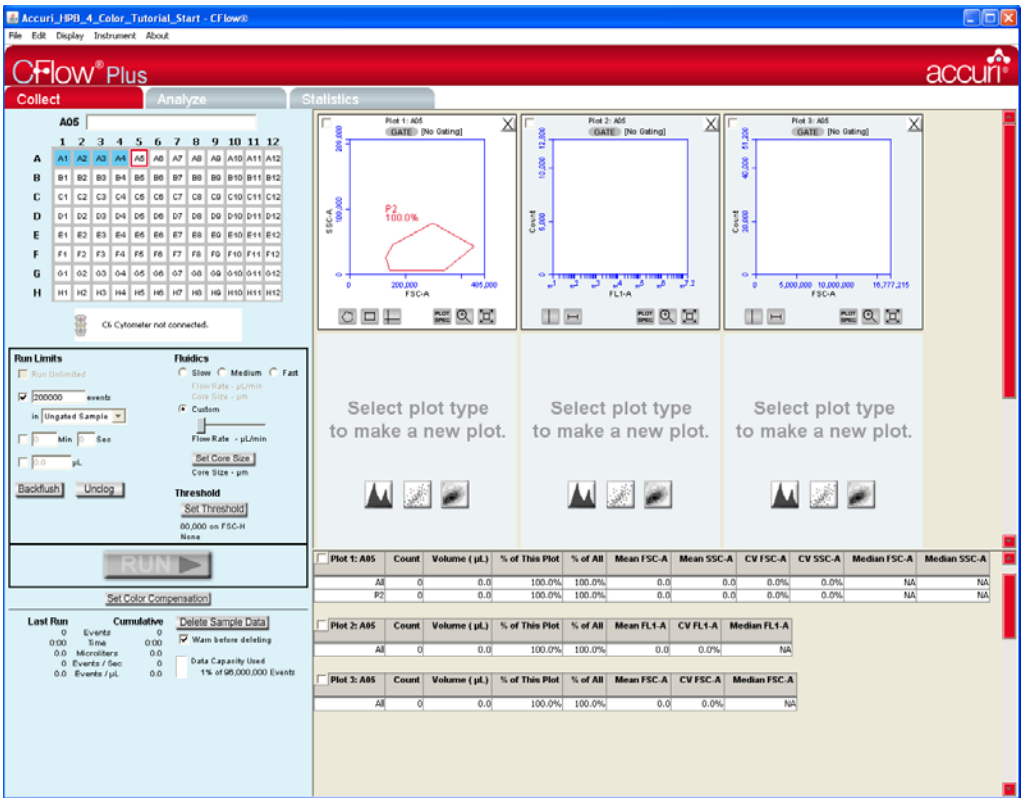


Figure 3-9. CFlow Workspace with Empty Sample Well and Empty Plots

4. Click on the *RUN* (or *ADD TO*) button to start a sample collection. CFlow displays and updates data in real-time during the collection. The C6 stops sampling from the tube when the run limit is reached.

   CAUTION: If you click on the *ADD TO* button, CFlow will collect data into a well that already contains data.

3.2.7 Pausing Data Collection

You can interrupt sample acquisition any time during a run. To stop a run:

1. Click on the *PAUSE* button.

To restart the run:

1. Click on the *ADD TO* button. CFlow resumes data collection in the current well.

3.3 Ending a Data Collection Session

When you finish collecting samples, rinse out the SIP to ensure cells or other particles are not left in the SIP.

To end a data collection session:

NOTE: If you plan to collect more samples later in the day, you can skip steps 1-8.

1. Place a tube with 2 mL of filtered, de-ionized water on the SIP and advance to any empty data well.
2. Set the time limit for two minutes.
3. Click on the *RUN* button.
4. Place a tube with 2 mL of decontamination solution (#KR-200) on the SIP.
5. Select an empty data well.
6. Set the time limit for two minutes and the fluidics speed to fast.
7. Click on the *RUN* button.
8. Once the run is finished, remove the tube of decontamination solution from the SIP.
9. Place a tube with 2 mL of filtered, de-ionized water on the SIP and advance to any empty data well.
10. Set the time limit for two minutes.
11. Click on the *RUN* button.
12. When the run is finished, leave the tube on the SIP.

3.4 Creating Plots

Plots allow you to view sample data in histogram, density, and dot graphs. You can view multiple plots for each sample you collect.

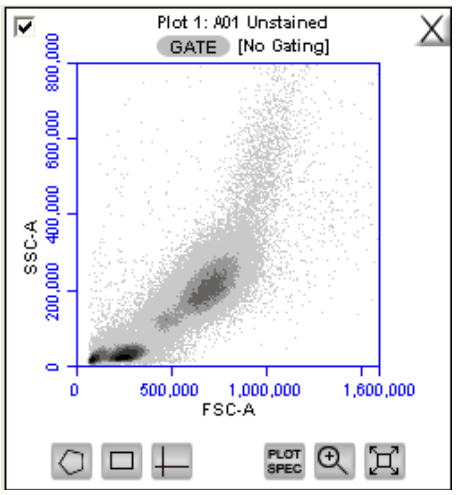


Figure 3-10. View of a Plot

Each plot contains a set of gating and marking tools and a set of viewing tools:

- Gating and marking tools:
  - *Gate* button GATE—Opens the Change Gating dialog box for applying gates to a plot.
  - Polygonal Gating Tool —Used for drawing irregularly shaped gates around a population of events.
  - Rectilinear Gating Tool —Used for drawing a rectilinear gate around a population of events.
  - Quadrant Gating Tool —Used for gating the plot in quadrants.
  - Vertical Marker Tool —Used for gating histograms to the right or left of a vertical marker.
  - Horizontal Marker Tool —Used for gating histograms within a horizontal marker.
- Viewing tools:
  - Plot Spec Tool —Opens the Set Plot Specs dialog box for changing the x- and y-axis parameters, scaling the plot, and setting log or linear view.
  - Zoom Tool —Allows you to draw a rectilinear range in the plot to zoom in on.
  - Expand Tool —Zooms out one level.

To create a new plot:

1. Click on one of the following icons in an empty plot corral:
   - *Density Plot*
   - *Dot Plot*
   - *Histogram Plot*

   CFlow displays an FSC-A vs. SSC-A plot (or FSC-A, for histogram) by default.

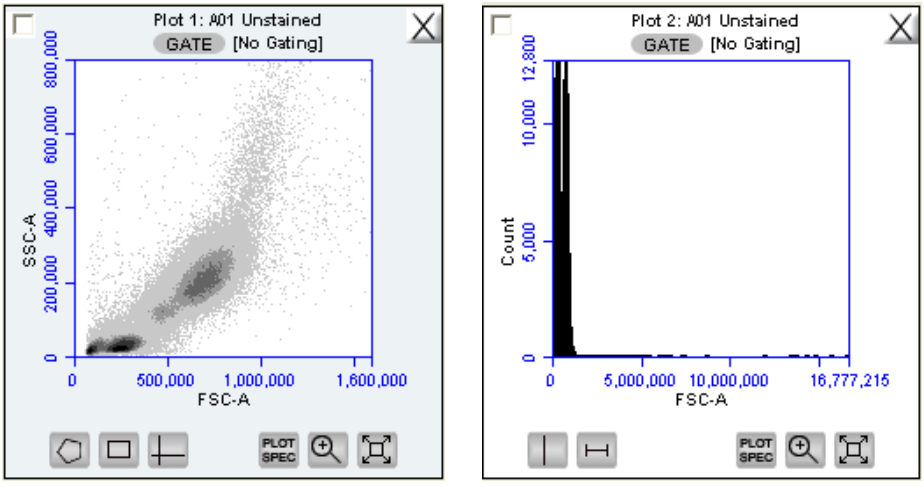


Figure 3-11. New Density and Histogram Plots

2. Configure the plot specifications as needed.

3.5 Changing Plot Specifications

The Plot Spec Tool allows you to change the way data are displayed in a plot. You can change axis parameters, specify channel ranges, and toggle between linear and logarithmic scales. The Plot Spec Tool is available in the *Collect* and *Analyze* tabs.

You can set up or modify plot specifications at any time before or after collecting data.

To change the plot specifications:

1. Click on the *Plot Spec Tool* icon.

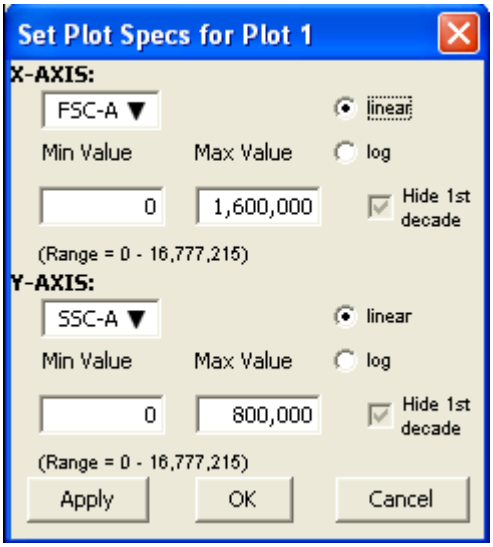


Figure 3-12. Set Plot Specs Dialog Box

2. In the Set Plot Specs dialog box, do the following for each axis:
   - Select the parameter you want to view in the parameter drop-down list.
   - Select the *linear* or *log* radio button to specify how data are displayed.
   - Type in the minimum and maximum channels to set the channel range to view.
   - Enable or disable the *Hide 1st decade* check box to indicate whether you want CFlow to display the first decade of channels in the plot.
3. Do one of the following:
   - Click on the *Apply* button to apply the changes without closing the dialog box.
   - Click on the *OK* button to apply the changes and close the dialog box.
   - Click on the *Cancel* button to close the dialog box without applying your changes.

3.6 Using Gates and Markers

A gate is a specified area within a plot that is used to designate a set of events to analyze. You can create any of the following types of gates:

- Polygonal gate—Gates an irregularly shaped area around a population of events.
- Rectilinear gate—Gates a rectilinear area around a population of events.
- Quadrant gate—Gates the plot in quadrants.
- Vertical marker—Gates a histogram plot to the right or left of a vertical marker.
- Horizontal marker—Gates a histogram plot within a horizontal marker.

3.6.1 Creating a New Gate

To create a gate in a density or dot plot:

1. Click on one of the following gating tools:
   - *Polygonal Gating Tool* —typically used for irregularly shaped populations.
   - *Rectilinear Gating Tool* —typically used for evenly shaped populations.
   - *Quadrant Gating Tool* —typically used for fluorescence compensation (see section 3.12 for details).
2. Use the mouse to draw a region (labeled *P1* for a polygonal gate, *R1* for a rectilinear gate, or *Q1* for a quadrant gate). If you are drawing a polygon, click on the mouse to anchor each vertex and double-click to close the polygon.

CFlow automatically displays the percentage of cells within the region.

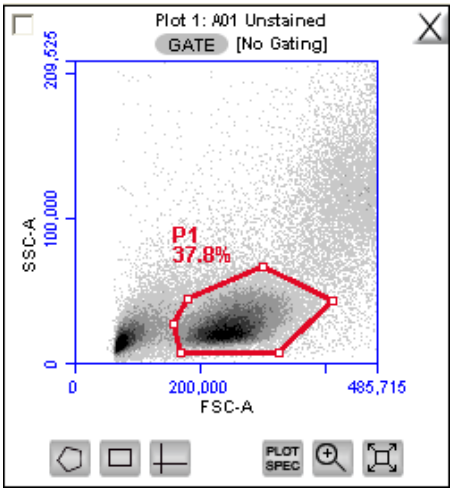


Figure 3-13. Using Polygonal Gating Tool

To create a vertical marker in a histogram plot:

1. Click on the *Vertical Marker Tool* .

2. Click the cursor at the point along the x-axis where you want to place the marker. CFlow automatically displays the percentage of cells to the left (*V1-L*) and right (*V1-R*) of the marker.

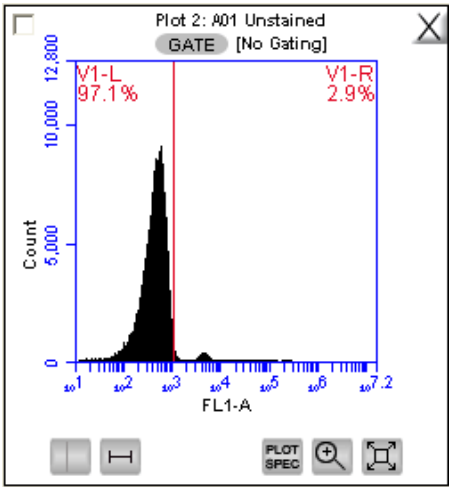


Figure 3-14. Using the Vertical Marker

To create a horizontal marker in a histogram plot:

1. Click on the *Horizontal Marker Tool* .
2. Click and drag the cursor horizontally across the area you want to gate. CFlow automatically displays the percentage of cells within the margins of the marker (labeled *M1*).

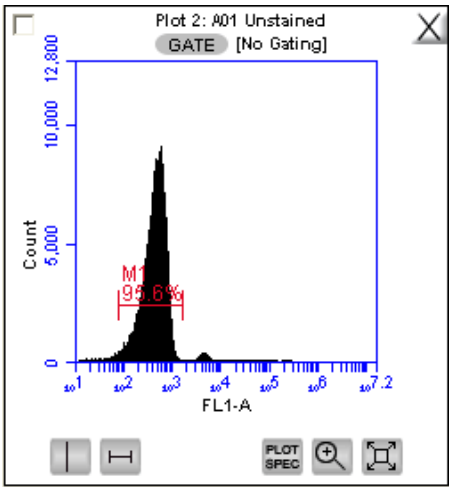


Figure 3-15. Using the Horizontal Marker

3.6.2 Applying a Gate to a Plot

To apply a gate to a plot:

1. Click on the *GATE* button at the top of the plot to which you want the gate applied.

2. Only polygon (P), rectilinear (R), and marker (M) gating regions automatically appear in the Gating dialog box list of options. If you want to view a list of vertical markers or quadrant markers, enable the associated check box(es) in the Change Gating dialog box.

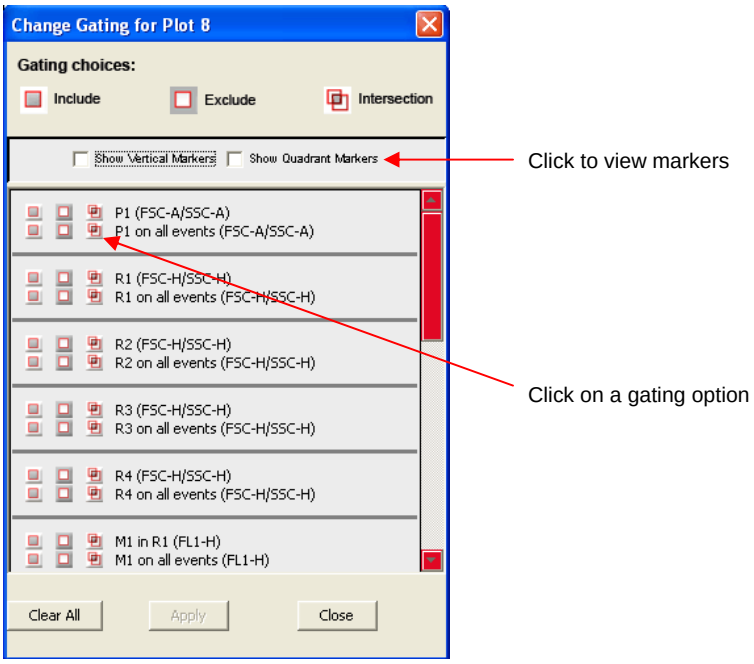


Figure 3-16. Selecting a Gating Option

3. Select one of the following gating icons associated with the gate you want to use:
   - *Include* icon —to analyze the events within the region. You can choose more than one gate with *Include* to analyze events in either one *or* the other gate.
   - *Exclude* icon —to analyze the events outside of the region.
   - *Intersection* icon —to analyze the events within the intersection of two or more regions (select this icon for each region you want to use).

4. Click on the *Apply* button. CFlow displays the type of gate that is applied next to the *GATE* button in the plot.

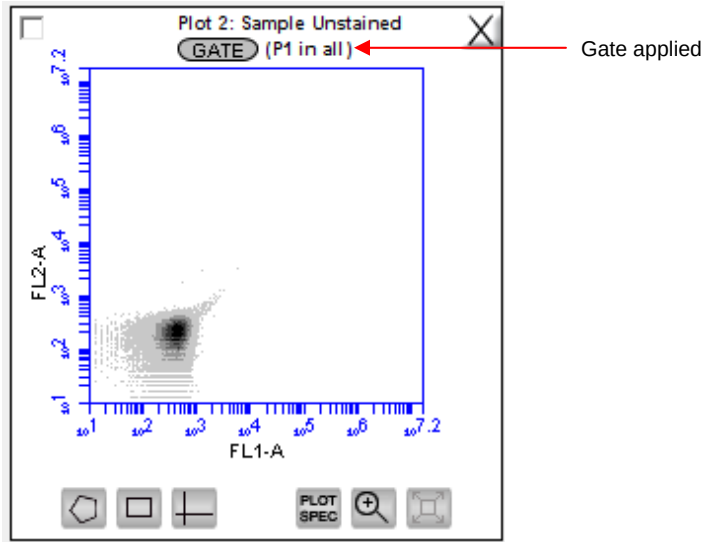


Figure 3-17. Plot Gated to Include P1

3.6.3 Creating and Applying Nested Gates

You can create a series of nested gates in which each gate is a subset of the previous one. This allows you to fine tune your gating strategy for viewing and analyzing specific subsets of data.

To create nested gates:

1. Draw any region or marker around a population of events.

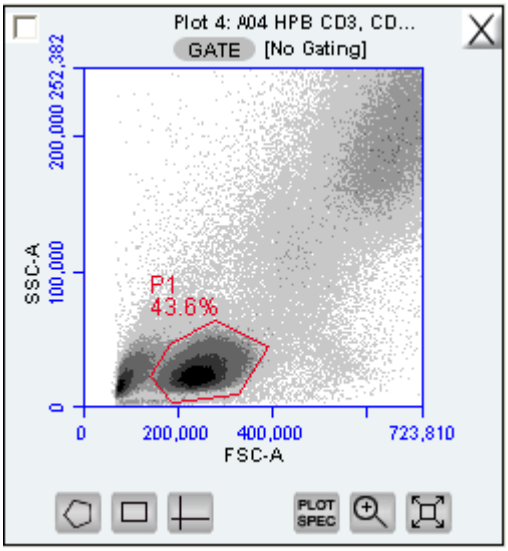


Figure 3-18. First Gate for Creating Nested Gates, Drawn in Plot 4

2. Click on the *GATE* button in a second plot and apply the gate. This is the *parent gate.*

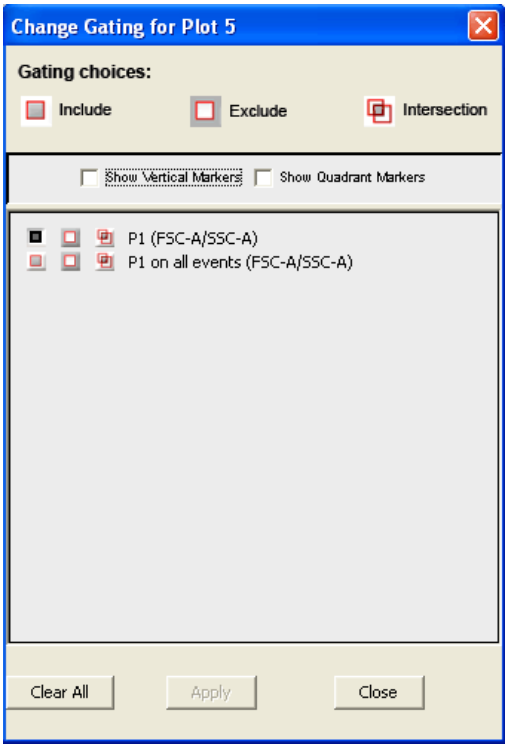


Figure 3-19. Applying the Parent Gate to Plot 5

3. Close the dialog box. The plot displays only the populations within the parent gate.

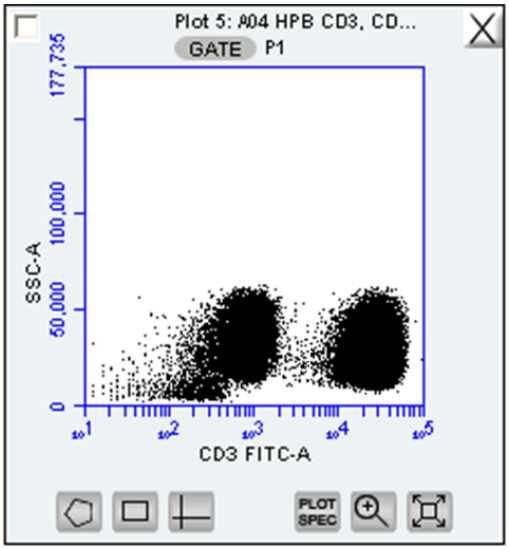


Figure 3-20. Parent Gate Applied to Dot Plot

4. In the plot that is gated on the parent gate (plot 5 in this example), draw a second region or marker around a subset of the population displayed in the plot.

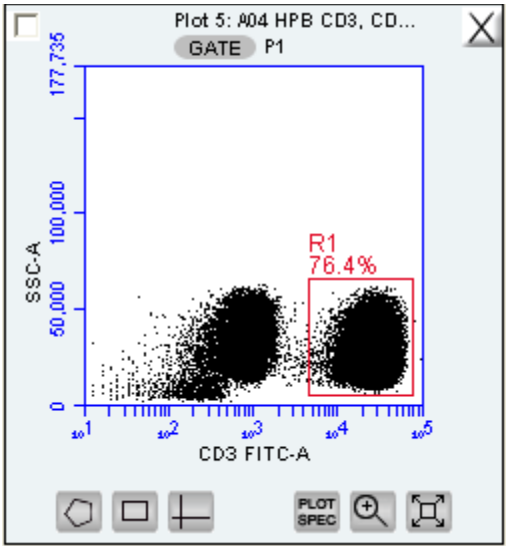


Figure 3-21. Second Gate for Creating Nested Gates

5. Open a third plot and click on the *GATE* button.
6. In the Change Gating dialog box, select the option in which the second gate is "*in*" the parent gate (see the following figure). Do not select the *on all events* option.

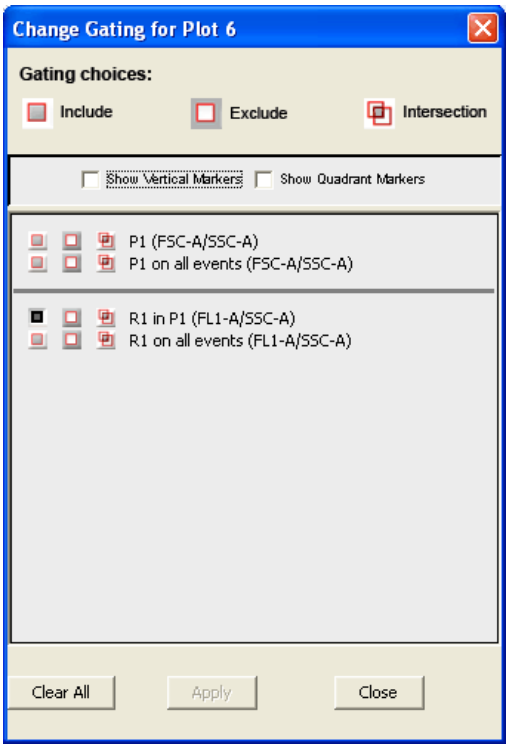


Figure 3-22. Applying the Child Gate

7. Apply the gate. This is the *child gate.*

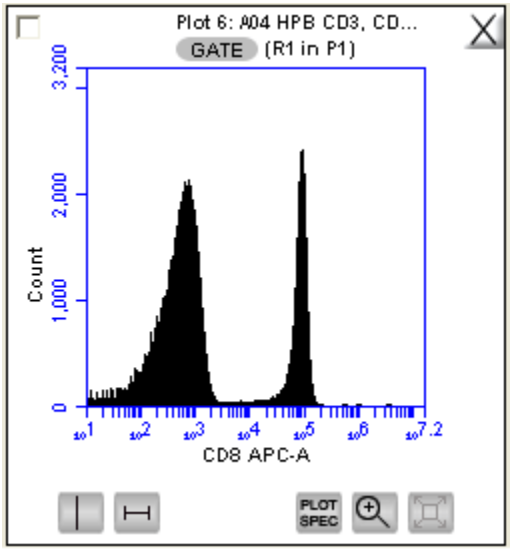


Figure 3-23. Third Plot with Nested Gate Applied (R1 in P1)

8. View the statistics in the Statistics Table.

Plot 6: A04 HPB CD3, CD4, CD45, CD8 Gated on (R1 in P1)	Count	Volume (µL)	% of This Plot	% of All	Mean CD8 APC-A	CV CD8 APC-A	Median CD8 APC-A
This Plot	76,415	0.0	100.00%	33.32%	18,572.85	211.93%	526.0

Figure 3-24. Statistics of Plot with Nested Gate

9. Close the dialog box.

3.7 Changing the Number of Events in a Plot

You can change the number of events displayed in all plots across all samples to make it easier to view the data. This option allows you to visually remove a number of events from the plot without deleting data.

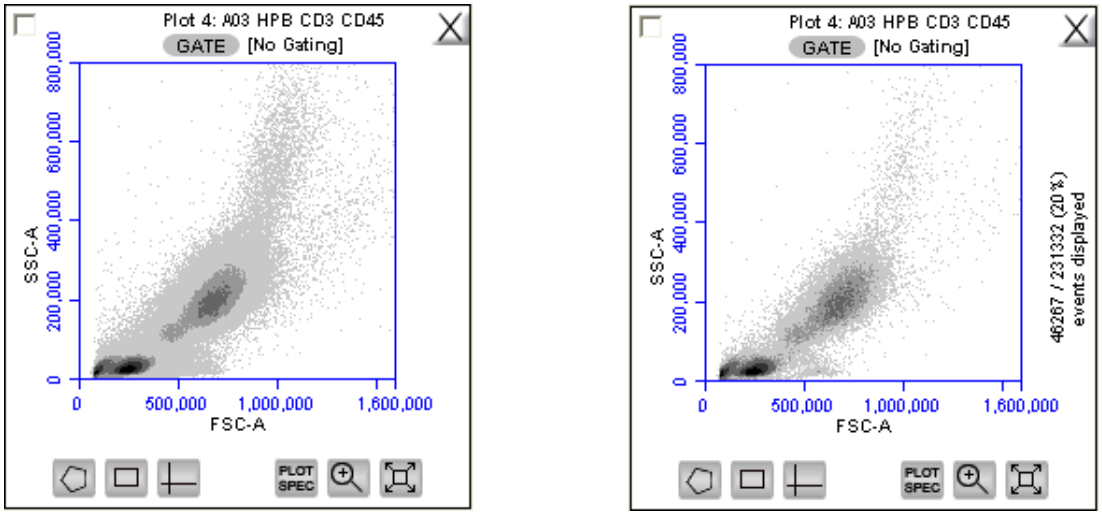


Figure 3-25. Before and After Changing Events Displayed

To change the events displayed in a plot:

1. Select *Display > Events Display Settings.*
2. Do one of the following in the Events Display Settings dialog box:
   - To view all collected events, select the *Show all events* radio button.
   - To view the first N events of a sample, select the *Display first* radio button and type a number in the *events collected* field.
   - To view a specified percentage of the whole in a pseudo-random selection, select the *Display* radio button and type a percentage to view (for example, if 20% is selected, every fifth event is displayed).

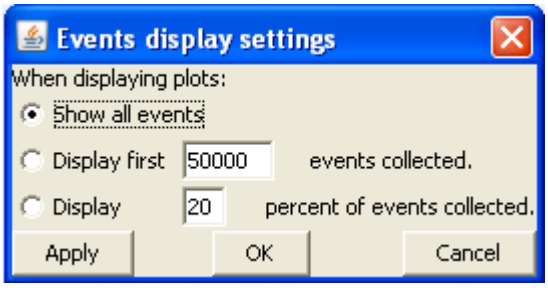


Figure 3-26. Events Display Settings Dialog Box

3. Do one of the following:
   - Click on the *Apply* button to apply your settings without closing the dialog box.
   - Click on the *OK* button to apply your settings and close the dialog box.
   - Click on the *Cancel* button to close the dialog box without applying your settings.

CFlow displays the plot and shows a message in the plot that some events are not being displayed.

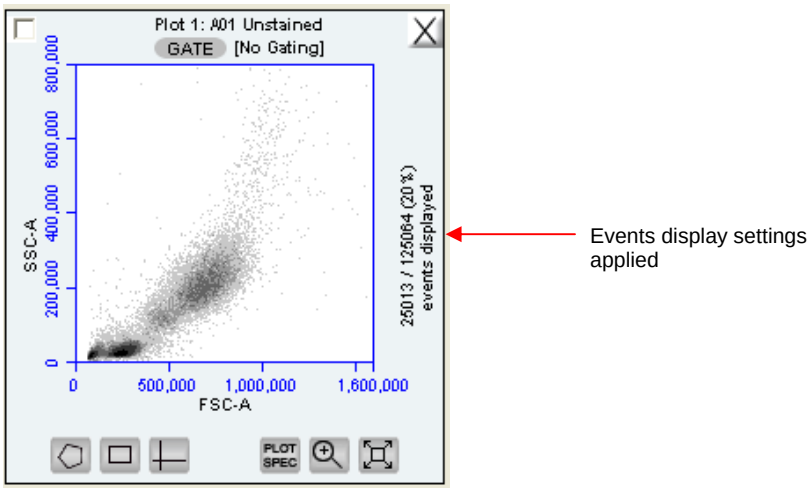


Figure 3-27. Plot with Events Display Settings Applied

3.8 Naming Plot Axes

You can rename the axis labels in a plot from the *Collect* or *Analyze* tab to identify the antibody staining or fluorochrome used in the sample.

To name a plot axis:

1. Click on an axis label and select *Rename Parameters* from the pop-up menu.

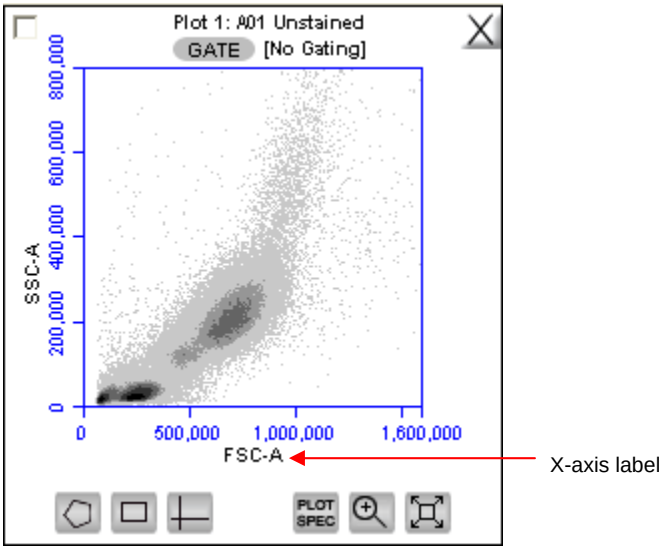


Figure 3-28. Click on an Axis Label

2. In the Rename Parameters dialog box, type the new label in the edit box of the parameter you want to rename.

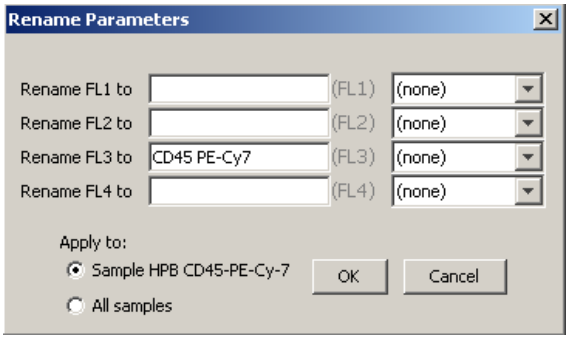


Figure 3-29. Rename Parameters Dialog Box

3. Do one of the following:
   - Select the *Sample XXX* radio button (where *XXX* refers to the current sample) to apply the label to the current sample only.
   - Select the *All Samples* radio button to apply the label to all samples.

To assign the name to the same parameter in another sample:

1. Select another sample from the 96-well grid.
2. Click on an axis label in a plot and select *Rename Parameters* from the pop-up menu.

3. Select the name from the drop-down list associated with the parameter.

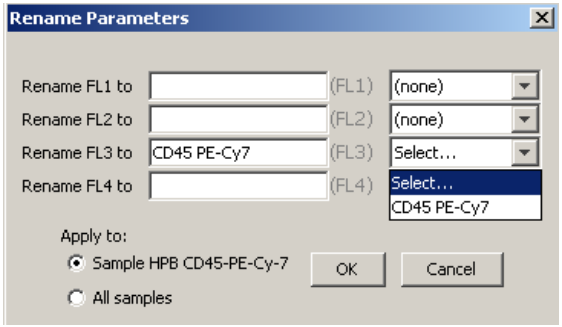


Figure 3-30. Rename Parameters Dialog Box with Axis Label Drop-Down List

3.9 Zooming on a Plot

CFlow automatically zooms the initial display of any parameter on a logarithmic scale from channel 10 to 16.7 x10^6. For most analyses, very few events fall into channels 0 to 10, so automatic zooming saves time by reducing the number of zoom steps. However, take care when setting markers (M), regions (R), or polygons (P) that require channels lower than 10 on a zoomed plot. It is recommended to unhide the first decade of data (see section 3.5) to prevent events from being excluded from gating regions, especially when setting fluorescence compensation (see section 3.12).

3.9.1 Basic Zoom

To zoom on a population:

1. Click on the *Zoom Tool* .
2. Click and drag the mouse in the plot to draw an area to zoom on (Figure 3-31).

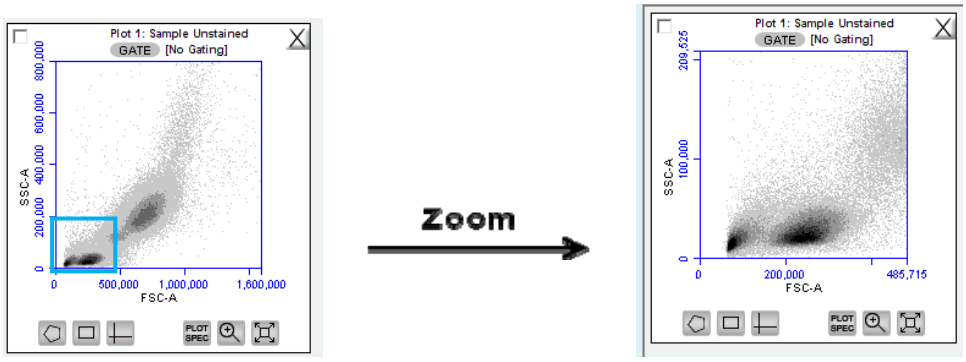


Figure 3-31. Before and After Using Zoom Tool

3. Repeat steps 1-2 as needed to zoom in closer.

To zoom out:

1. Click on the *Expand Tool* .
2. Repeat step 1 as needed.

3.9.2 Zooming to a Specified Channel Range

Sometimes it can be helpful to view a plot in a specified channel range.

To view a specified channel range in a plot:

1. Click on the Plot Spec Tool in the plot you want to zoom.

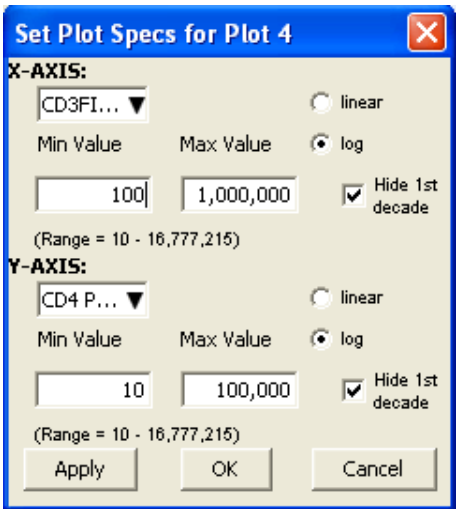


Figure 3-32. Plot Spec Dialog Box: Set Min and Max Channel Values for the X- and Y-Axes

2. Specify the x-axis channel range by typing a minimum (*Min Value*) and maximum (*Max Value*) value under *X-Axis* in the Set Plot Specs dialog box.
3. Specify the y-axis channel range by typing a minimum and maximum value under *Y-Axis*.
4. Click on the *Apply* button to apply the changes and click on the *OK* button to close the Plot Spec dialog box.

3.10 Saving a CFlow File

Always save C6 data as a CFlow file (.c6). A CFlow file is a comprehensive (and often large) data file that contains instrument settings, FCS files, and plot layouts.

The CFlow file contains the entire CFlow workspace, including the following elements:

- Sample data
- Plot layouts
- Gating
- Color compensation
- Threshold settings
- *Collect* tab settings
- Changes made in the *Analysis* or *Statistics* tabs

By default, CFlow automatically saves CFlow data at the end of each sample run. You can also manually save data at any time. To save the entire CFlow file, save the file manually (see section 3.10.2).

When a CFlow file is saved, CFlow displays the file name in the upper left corner of the workspace (Figure 3-33).

Figure 3-33. Title Bar with File Name

3.10.1 Auto-Saving Files

By default, CFlow automatically saves the event data any time the C6 reaches a run limit or if you click on the *Pause* button during a run. Auto-save *does not* save acquisition settings, plots, or gating strategies.

CAUTION: If you make changes after a run or after pausing a run, CFlow *does not* automatically save the file. Save these changes manually (see section 3.10.2).

To enable or disable auto-save:

1. Select *File* > *Auto-save Settings*.
2. Do one of the following in the Auto-save Settings dialog box:
   - Select the *Auto-save Enabled* radio button to enable auto-save.
   - Select the *Auto-save Disabled* radio button to disable auto-save.

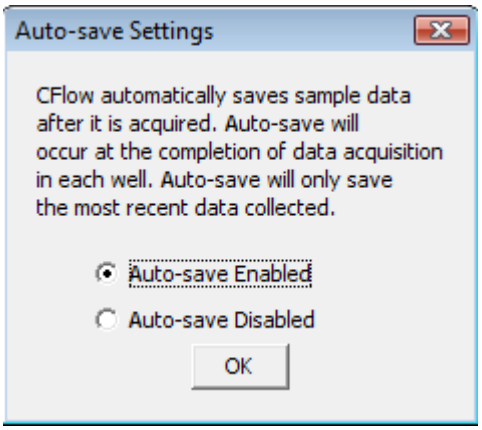

Auto-save Settings
CFlow automatically saves sample data after it is acquired. Auto-save will occur at the completion of data acquisition in each well. Auto-save will only save the most recent data collected.
Auto-save Enabled
Auto-save Disabled
OK

Figure 3-34. Auto-Save Settings Dialog Box

3. Click on the *OK* button to accept the change and close the dialog box.
4. If prompted to save the workspace before closing, do one of the following:
   - Click on the *Yes* button to save the entire workspace.
   - Click on the *No* button to exit the dialog box without saving the workspace.

3.10.2 Manually Saving Files

You can manually save a CFlow file at any time.

To manually save a CFlow file:

1. Select *File* > *Save.*

To manually save a CFlow file with a new name:

1. Select *File* > *Save CFlow File As.*

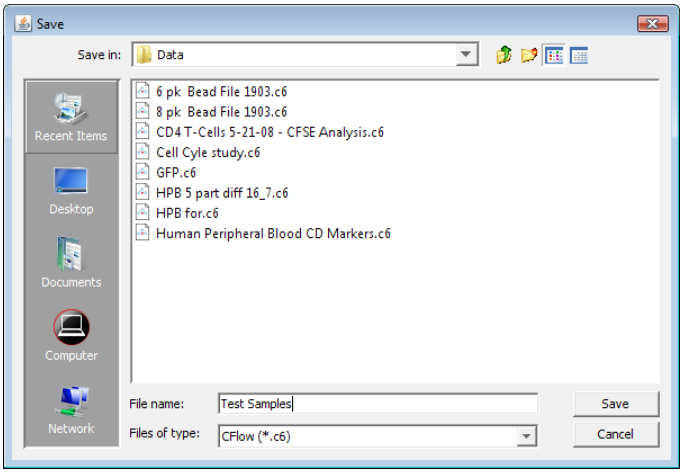


Figure 3-35. Save CFlow File

2. If necessary, navigate to the location to save the file.
3. In the Save dialog box, enter the file name and click on the *Save* button. The file is saved with the extension .c6.

3.11 Creating a CFlow Template

A CFlow template contains a predefined CFlow workspace for quick and easy setup and analysis. All markers, regions, gates, parameter names, and sample names are saved without any data points. Accuri provides several templates (see the CFlow Installation CD (or flash drive) or the Accuri website at www.accuricytometers.com/technical_information/templates/) or you can create your own customized templates.

To create a template:

1. Define plot, gating, and acquisition settings in a blank workspace, or use the current .c6 file.

2. Select *File* > *Save CFlow template as*.

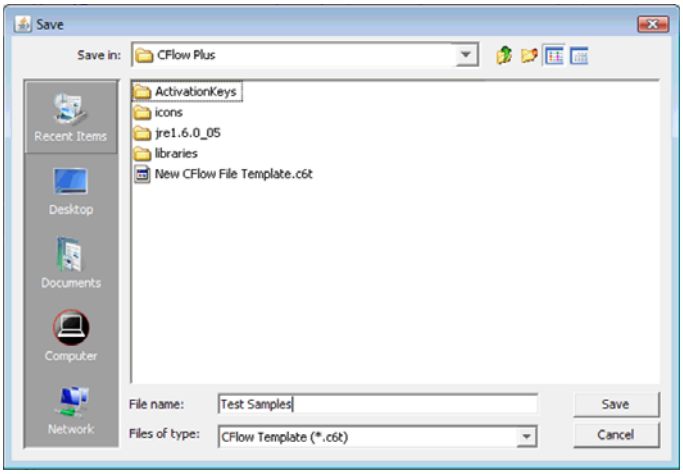


Figure 3-36. Save CFlow Template

3. If necessary, navigate to the location to save the file.
4. In the Save dialog box, enter the file name and click on the *Save* button. CFlow saves the file with the extension .c6t.

   NOTE: Acquisition settings are saved based on the currently selected sample.

3.12 Understanding Fluorescence Spillover

Fluorochromes typically emit light over a broad range of wavelengths, resulting in the fluorescence signal appearing not only in the expected, primary detector of a cytometer but in other detectors as well. This phenomenon is often called fluorescence “spillover,” and can be a source of confusion when interpreting multi-color flow cytometric data.

3.12.1 Recognizing Fluorescence Spillover

Whenever performing a multi-color experiment, prepare a set of control samples, each stained with one individual fluorochrome. These single-stained controls will allow you to determine the extent of fluorescence spillover from each fluorochrome. The example shown in the figure below shows data collected for a PE-Cy7 single-stained control. Most of the fluorescence signal from PE-Cy7 positive cells was detected in the FL3 (670 LP), as expected. However, there is also PE-Cy7 signal detected in FL1 (530 BP) and FL2 (585 BP), so that plots of data for those detectors appear to have positively fluorescent cells. No signal from PE-Cy7 appears in detector FL4.

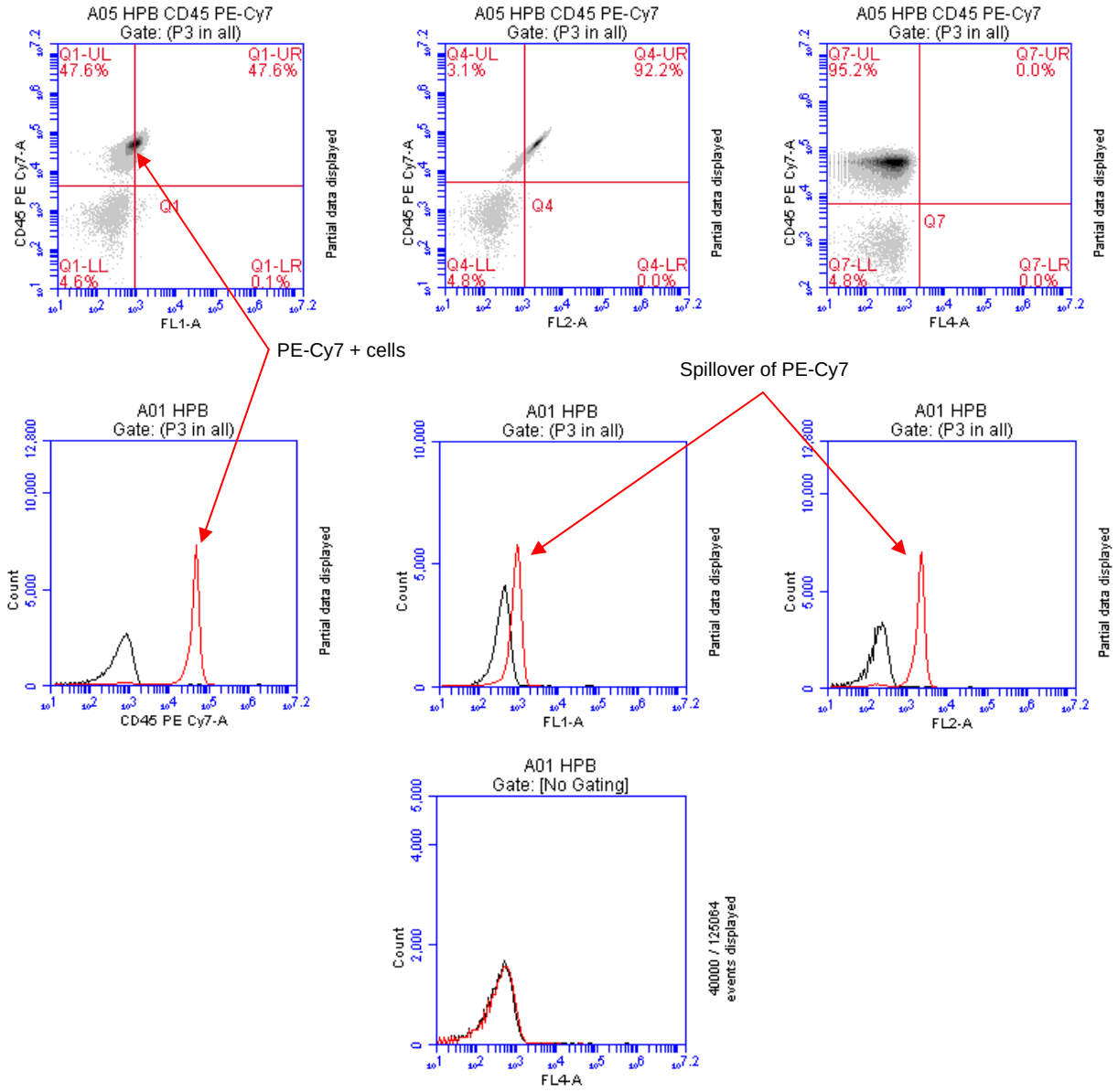


Figure 3-37. Fluorescence Spillover in Different Plots

3.12.2 Correcting Fluorescence Spillover

You can remove fluorescence spillover from plots by applying a mathematical algorithm to collected data. This process is often called *color compensation* (or *fluorescence subtraction*). Because data collection on the C6 is digital, color compensation can be applied or removed before, during, or after data collection. The color compensation algorithm subtracts a user-defined percentage of fluorescence signal from every event, thereby redistributing data to lower channels of the fluorescence scale and removing the apparent fluorescence spillover. When color compensation has been properly applied to a data set, the median fluorescence channel value in non-primary detectors for any given single-stained control sample should be the same as that of an unstained control sample.

The following figure shows the data after proper color compensation has been applied.

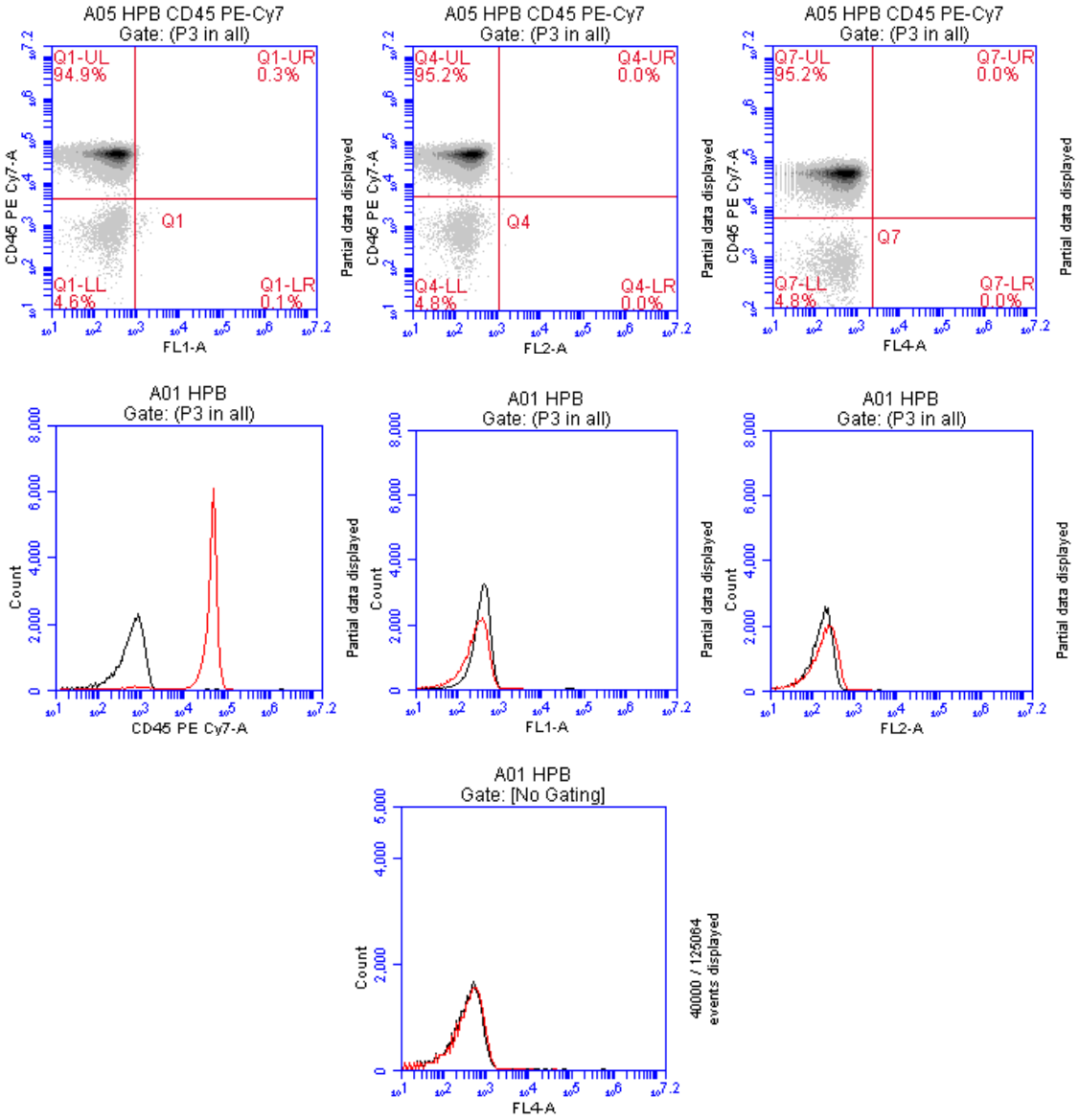


Figure 3-38. Corrected Fluorescence Spillover

To correct fluorescence spillover:

1. Click on the Quadrant Tool of a plot you want to correct and click inside the plot.

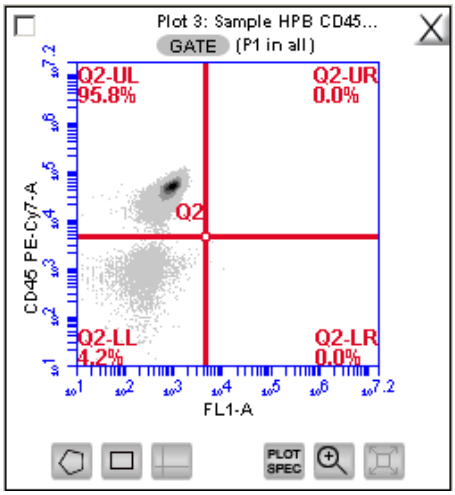


Figure 3-39. Placing a Quadrant Tool

2. Adjust the quadrant marker position so that all positive populations are cleanly contained in individual quadrants.

   CFlow displays the median fluorescence channel value for the events in each quadrant in the Statistics Table (shown below). No median is calculated for the line named *All* or *This Plot* because this line contains summary data for the entire plot.

Plot 3: Sample A2 Gated on (P1 in all)	Count		Median FL1-A	Median CD45 PE-Cy7-A
This Plot	101,660	%	NA	NA
Q1-UL	96,737	%	841.0	45,399.0
Q1-UR	0	%	0.0	0.0
Q1-LL	4,921	%	240.0	771.0
Q1-LR	2	%	10,200.0	1,322.0

Figure 3-40. Statistics Table Displaying Median Values

3. Compare the median values of the affected channel. If the median value of the UL or LR quadrant is not equal to the median value of the negative population (LL), you will need to perform fluorescence compensation.

4. Click on the *Set Color Compensation* button in the *Collect* or *Analyze* tab to open the *Compensation Settings* dialog box. The dialog box contains four rows of *FL* buttons, one row for each fluorescence channel.

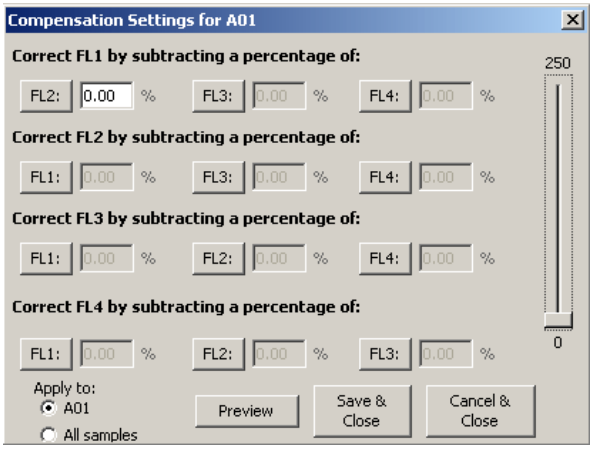


Figure 3-41. Compensation Settings Dialog Box

5. In the row associated with the channel to correct, click on the *FL* button of the fluorescence channel that is spilling over.
6. Do one of the following:
   - In the text box next to the *FL* button, type an arbitrary percentage of the signal to subtract.
   - Use the C Comp Calculator Excel spreadsheet provided by Accuri (available on the CFlow installation CD (or flash drive) or at www.accuricytometers.com/technical_information/Brochures/manuals/) to calculate the subtraction values. The spreadsheet is more accurate and saves time.
   - Use the following table as a guide:

Table 3-2. Fluorochrome Spillover per Channel

		Spillover Fluorochrome					
		FITC	**PE**	**PerCP**	**PerCPCy5.5**	**PE-Cy7**	**APC**
Channel to Correct	**FL1** (530BP)	N/A	3.5	0.00	0.0	1.00	N/A
	FL2 (585 BP)	7.0	N/A	0.00	0.00	3.50	0.0
	FL3 (670 LP)	1.0	14.5	N/A	N/A	N/A	1.2
	FL4 (675 BP)	0.0	0.0	3.00	12.00	0.00	N/A

7. Click on the *Preview* button to update the Statistics Table.
8. In the Statistics Table, observe the values in the *Median* column of the FL channel you are correcting.

9. Repeat steps 5-8 until the the median value for the UL or LR quadrant is equal (or nearly equal) to the median value of the negative population (LL). This value is called the *compensation value*. The figure below shows the median values highlighted in blue.

me (µL)	% of This Plot	% of All	Mean FL1-A	Mean CD45 PE-Cy7-A	CV FL1-A	CV CD45 PE-Cy7-A	Median FL1-A	Median CD45 PE-Cy7-A
0.0	100.0%	44.2%	253.9	42,578.8	72.6%	35.8%	NA	NA
0.0	95.2%	42.0%	252.9	44,700.5	70.2%	27.6%	238.0	45,399.0
0.0	0.0%	0.0%	0.0	0.0	0.0%	0.0%	0.0	0.0
0.0	4.8%	2.1%	269.9	887.2	87.6%	71.3%	231.0	771.0
0.0	0.0%	0.0%	7,973.0	1,145.5	27.8%	15.4%	10,187.0	1,322.0

Compare medians

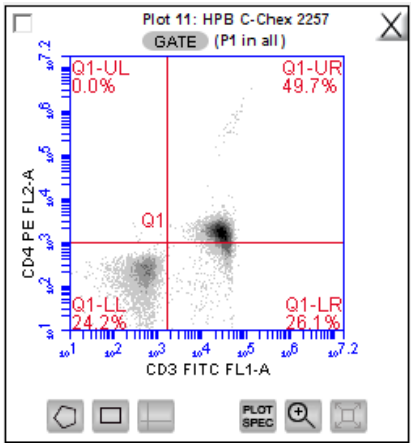
Figure 3-42. Results of Subtracting Spillover

10. If you want to apply the fluorescence subtraction to all samples, select the *Apply to All samples* radio button in the Compensation Settings dialog box.
11. Click on the *Save & Close* button to apply the color compensation settings.

3.12.3 Troubleshooting Color Compensation

Occasionally, the plot might appear to have a smaller percentage of events in a quadrant than CFlow reports as present. For example, Figure 3-43 appears appears to have less than 26.1% of the population in the lower right quadrant, even though the statistics report 26.1%. This occurs when a decade containing data is hidden.

In the following figure, a number of events have been driven into channel 1 because of overcompensation. These events are not displayed in the plot if the first decade is hidden, but they are included in statistics calculations.



Plot 11: HPB C-Chex 2257 Gated on (P1 in all)	Count	Volume (µL)	% of This Plot
This Plot	21,479	73.3	100.0%
Q1-UL	7	73.3	0.0%
Q1-UR	10,669	73.3	49.7%
Q1-LL	5,198	73.3	24.2%
Q1-LR	5,605	73.3	26.1%

Figure 3-43. Plot Displaying Overcompensation

To fix overcompensation:

1. Click on the Plot Spec Tool .

2. In the Set Plot Specs dialog box, disable the *Hide 1st decade* check boxes for both X- and Y-axes.

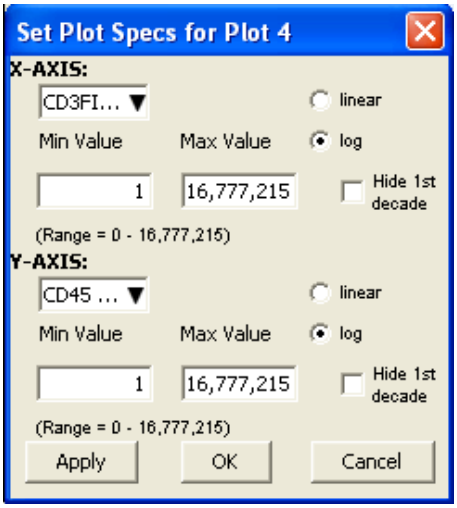
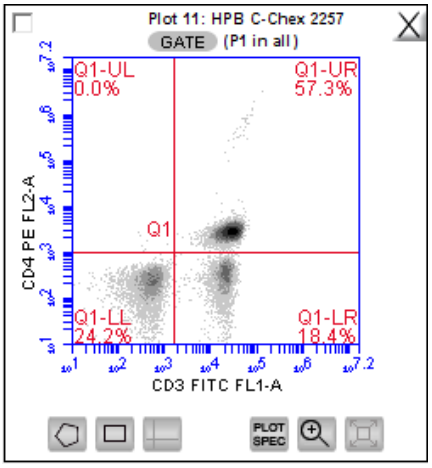


Figure 3-44. Set Plot Specs Dialog Box with *Hide 1st decade* Disabled

3. Click on the *OK* button to apply the settings and close the dialog box.
4. Perform the fluorescence compensation procedure described in section 3.12.2. The number of events displayed in Q1-LR agrees with the percent value of 18.4%, as shown on the plots and Statistics Table in Figure 3-45.



Plot 11: HPB C-Chex 2257 Gated on (P1 in all)	Count	Volume (µL)	% of This Plot
This Plot	21,479	73.3	100.0%
Q1-UL	8	73.3	0.0%
Q1-UR	12,315	73.3	57.3%
Q1-LL	5,197	73.3	24.2%
Q1-LR	3,959	73.3	18.4%

Figure 3-45. Properly Compensated Data After First Decade Is Rehidden

3.13 Changing Parameters

You can change the parameters that a plot displays along the x- and y-axes. By default, CFlow displays the area parameter (signified with a suffix of *-A*), but you can also choose a height, width, or time parameter.

To change a parameter:

1. Click on the x- or y-axis label and select the option you want from the pop-up menu.

NOTE: The time parameter starts counting when you click on the *Run* button and continues counting for 19 days (16 million tenths of a second), even if you add data to the sample at a later time. You cannot reset the parameter to zero, even by deleting data.

3.14 Copying and Pasting Plots

To copy and paste plots from the *Collect* or *Analyze* tab to a Microsoft® Office compatible application:

1. Click anywhere on a plot and drag it to an open Microsoft application. You cannot use **Ctrl+C** and **Ctrl+V** to copy and paste plots from CFlow into other applications.

3.15 Printing Data

You can print selected plots and their associated statistics from the *Collect* or *Analyze* tab.

To print plot data:

1. Enable the check box in the upper left corner of each plot that you want to print.

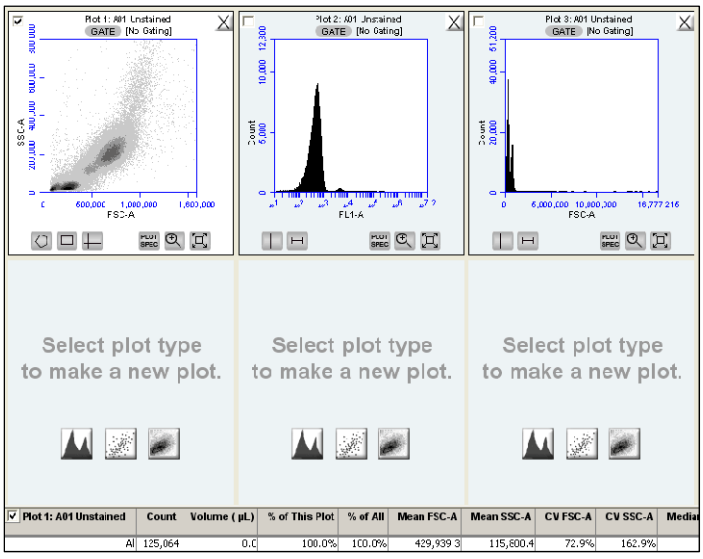


Figure 3-46. Plots Selected for Printing

2. If you do not want to print the associated Statistics Tables, disable the check box in the upper left corner of each table.

3. Select *File* > *Print Selected Items*.

3.16 Exporting and Importing Files

You can export data from individual sample wells from the *Collect* or *Analyze* tab as FCS 3.0 files at any time.

To export data:

1. Do one of the following:
   - Select *File* > *Export FCS File* to export and save the currently selected data well as an FCS 3.0 file.
   - Select *File* > *Export ALL Samples as FCS* to export and save all data wells as individual FCS 3.0 files.
   - Select *File* > *Export ALL Samples to Third Party* to export and save all data wells as individual FCS 3.0 files that enable autoscaling in third party applications (such as FlowJo).
   - Select *File* > *Export Plot Data as CSV* to save an individual file in .csv format.
2. If prompted to confirm the export, click on the *OK* button.

To import an FCS data file into CFlow:

1. Select an empty data well in a CFlow file or template.
2. Select File > Import FCS File.
3. Navigate to the location of the file.

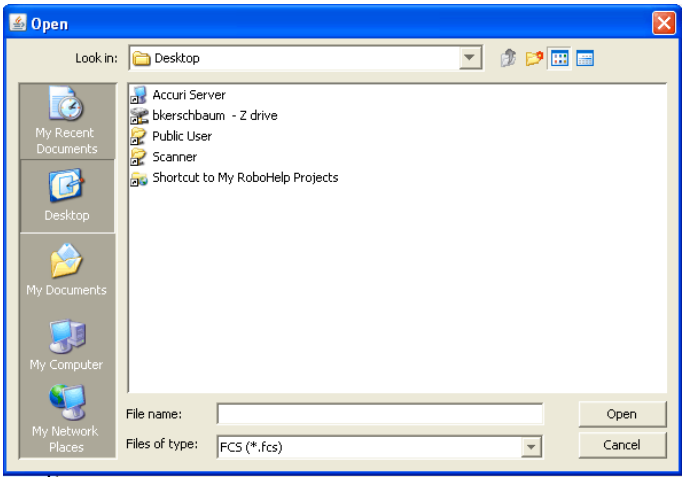


Figure 3-47. Open an FCS file

4. Select the file and click on the *Open* button.

4 ANALYZING SAMPLE DATA

The *Analyze* tab allows you to simultaneously view data from multiple samples using the same plots and gating.

Use the tab to do the following:

- View several plots and samples in any combination for easy analysis.
- Compare specific samples from the 96-well grid.
- Create new plots, hide or delete plots, or copy and reuse plots from the *Collect* tab.
- View different samples with the same plots.
- Create color overlay histograms.
- Print multiple plots.
- Adjust peak position.
- Calculate median statistics.

4.1 Viewing the *Analyze* Tab

The *Analyze* tab is organized into two major sections:

- Setup panel—Panel on the left side of the window that contains controls for selecting samples and plots.
- Data display—Large area on the right side of the window that shows sample data in plots and in a Statistics Table.

When you open the *Analyze* tab for the first time, the workspace is empty. To set up the *Analyze* tab, you can copy plots from the *Collect* tab, create new plots, and set up gating strategies.

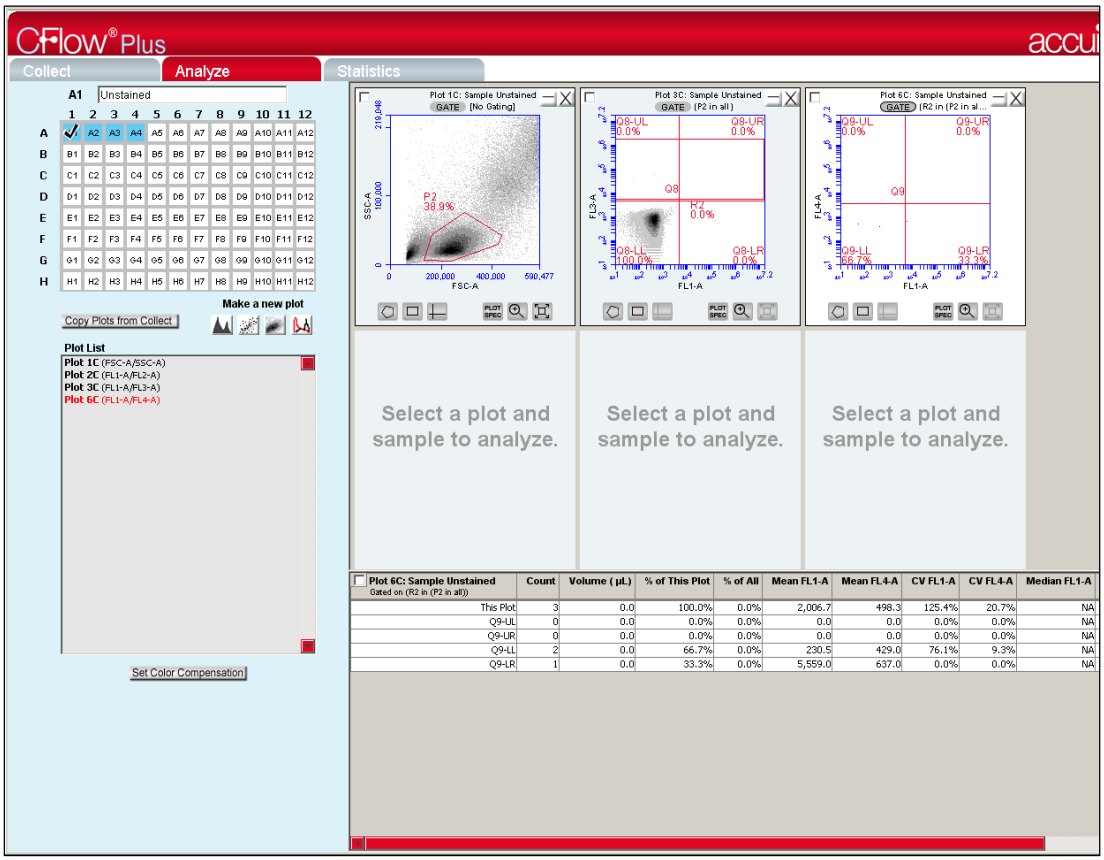


Figure 4-1. *Analyze* Tab Workspace

The following table describes each of the controls and indicators in the *Analyze* tab:

Table 4-1. *Analyze* Tab Controls

Control	*Description*
Sample Naming Field	Text box for naming the current sample.
Sample Grid	Matrix laid out in the configuration of a 96-well plate to help organize sample data. Each sample has its own well in the Sample Grid. The wells are color-coded: • White—Does not contain data. • Blue—Contains data. • Black check mark—Currently selected for viewing data.
Copy Plots from Collect	Copies specified plots from the *Collect* tab. See section 4.2.1 for details.

Control	*Description*
Plot Controls	Set of buttons for creating new plots or overlaying histograms. See section 4.2.3 for details. All plots created in the *Analyze* and *Collect* tabs, including overlays, can be dragged and dropped into most Microsoft Office compatible programs.
Plot List	Lists the plots that are available in the *Analyze* tab. Available plots include plots copied from the *Collect* tab or created in the *Analyze* tab.
Set Color Compensation	Opens the Color Compensation Matrix for correcting fluorescence spillover. See section 3.12 for details.
Plot Corrals	Area displaying two rows of plot corrals. Scroll up or down to view more plots. For information on creating plots, see section 4.2.2.
Statistics Table	Table below the plots that displays statistical information on individual plots. Statistics Tables can be copied into most Microsoft Office compatible programs.

4.2 Setting up Plots

In the *Analyze* workspace, you can copy plots from the *Collect* tab or create new plots. Plots that are copied from the *Collect* tab are appended with a “C” (for example, *Plot 1C*).

4.2.1 Copying Plots from the *Collect* Tab

To copy plots:

1. In the *Analyze* tab, click on the *Copy Plots from Collect* button.
2. In the Copy Plots from Collect dialog box, do one of the following:
   - Select the check boxes of the plots you want to copy.
   - Enable the *All Plots* check box to copy all of the plots from the *Collect* tab.

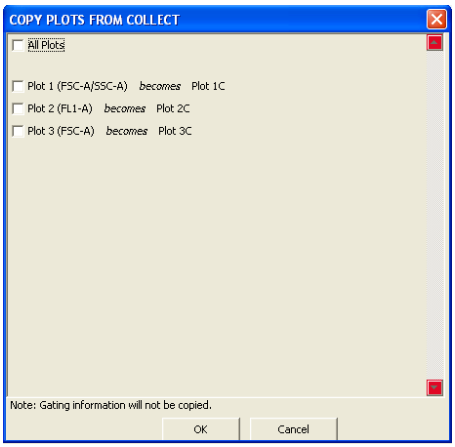

COPY PLOTS FROM COLLECT
All Plots
Plot 1 (FSC-A/SSC-A) becomes Plot 1C
Plot 2 (FL1-A) becomes Plot 2C
Plot 3 (FSC-A) becomes Plot 3C
Note: Gating information will not be copied.
OK
Cancel

Figure 4-2. Selecting Plots to Copy from the *Collect* Tab

3. Click on the *OK* button to close the dialog box. CFlow adds the selected plots to the Plot List in the *Analyze* tab (Figure 4-3).

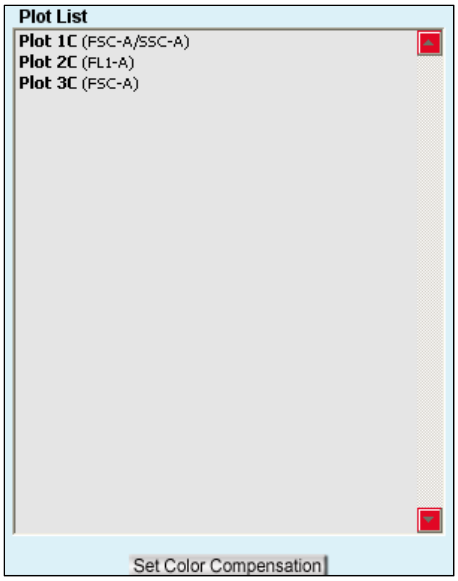


Figure 4-3. Plot List Containing Copied Plots

4.2.2 Creating Plots

To create a new plot:

1. Click on an empty plot corral.
2. Click on one of the following icons under the Sample Grid:
   - *Histogram*
   - *Dot*
   - *Density*
   - *Overlay Histogram* (see section 4.2.3 for details)
3. Click on the sample well that contains the data you want to view.

4.2.3 Creating an Overlay Histogram

You can compare multiple distributions from different samples at the same time by creating an overlay histogram.

To create an overlay histogram:

1. Click on an empty plot corral.

2. Click on the Overlay Histogram Tool to open a blank single-parameter FSC-A plot (Figure 4-4).

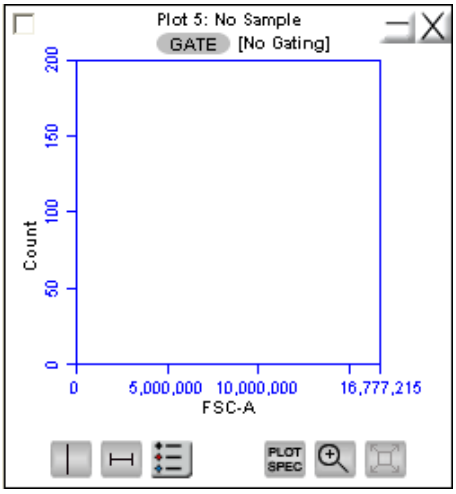


Figure 4-4. Blank Overlay Histogram Plot

3. Click on the x-axis label *(FSC-A)* and select a different parameter in the pop-up list, if desired.
4. Click on the *GATE* button and apply a gate as appropriate (see section 3.6 for details).
5. Select the data wells to be overlaid from the 96-well grid.

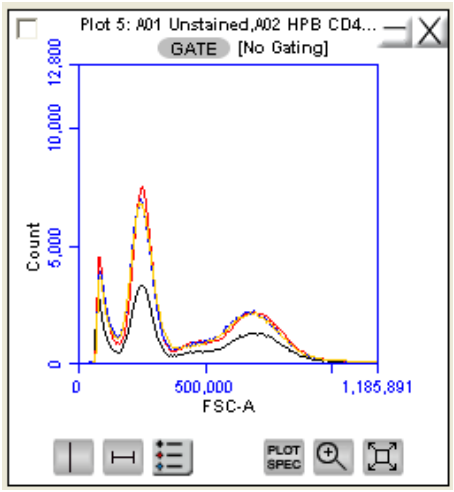


Figure 4-5. Overlay Histogram Plot with Data

6. Click on the Overlay Histogram Legend Tool to view a legend for the overlay histogram.

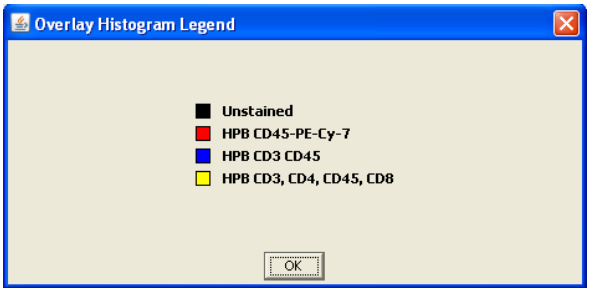


Figure 4-6. Overlay Histogram Legend

4.3 Viewing Plots

To view a plot in the Plot List:

1. Click on an empty plot corral in the *Analyze* tab.
2. Click a plot in the Plot List (Figure 4-7) to open the plot. CFlow displays the plot without any sample data.

   NOTE: Any gates copied from the *Collect* tab are renamed (for example, P1 in *Collect* is P2 in *Analyze*). These gates can be adjusted in the *Analyze* tab without changing the position of the gates in *Collect*.
3. Click on the well of the sample you want to view.
4. Apply a gating strategy, if desired (see section 3.6.2 for details).

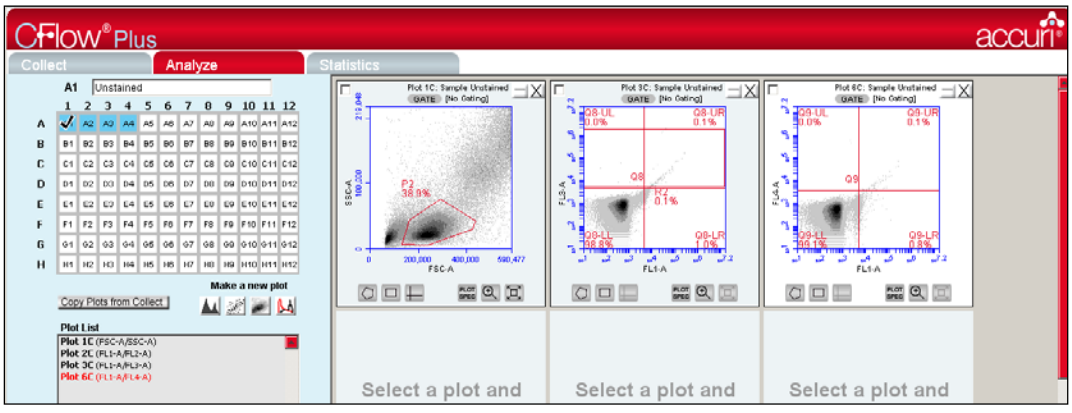


Figure 4-7. *Analyze* Tab: Plots with Gating Applied

5. To view data from another sample, open one or more plots from the Plot List (it is recommended to do this in another row of plot corrals) and choose the sample to be displayed in each plot. The gates that you applied above are automatically applied to the corresponding plots in the new row.

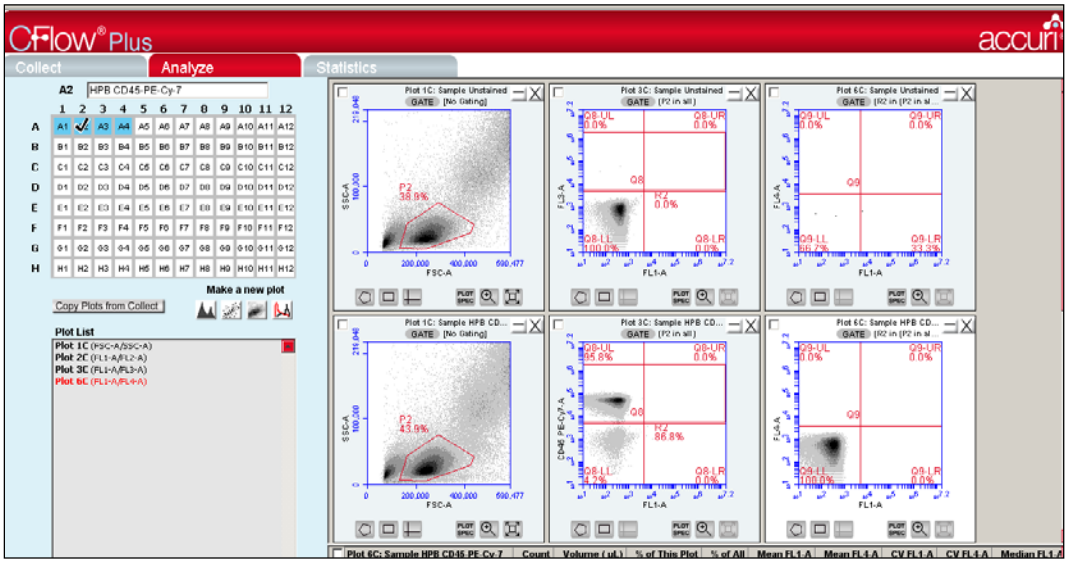


Figure 4-8. *Analyze* Tab: Two Samples Displayed with the Same Plots

6. Compare data and statistics between samples.

5 VIEWING STATISTICS

The *Statistics* tab provides a way of tabulating data from multiple samples in one master table. It also allows you to do the following:

- View statistics for some or all of your collected samples.
- Display statistics of collected or imported samples.
- List all plots created on the *Collect* and *Analyze* tabs.
- Display all plot names, gates, and associated statistics.
- Cut and paste data into a spreadsheet.

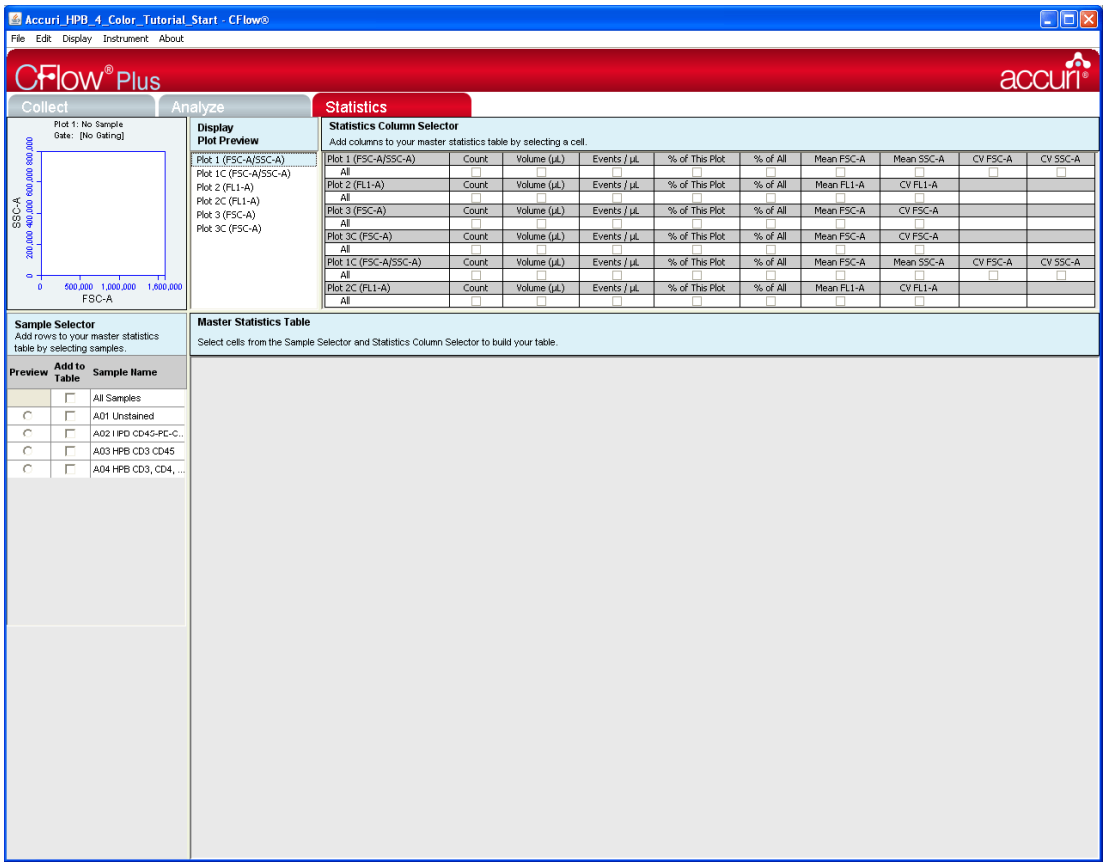


Figure 5-1. *Statistics* Tab Workspace

5.1 Viewing the *Statistics* Tab

The following table describes each of the controls and indicators in the *Statistics* tab:

Table 5-1. *Statistics* Tab Controls

Control	*Description*
Plot	Previews the selected plot. See section 5.3 for details.
Display Plot Preview	List of plots imported from the *Collect* and *Analyze* tabs that allows you to select the plot to preview.
Statistics Column Selector	Allows you to select the data to view in the Master Statistics Table for each sample. See section 5.2 for details.
Sample Selector	Allows you to select the samples to view in the Master Statistics Table. See section 5.2 for details.
Master Statistics Table	Configurable table that displays data of selected samples. See section 5.2 for details.

5.2 Creating the Master Statistics Table

The Master Statistics Table allows you to immediately view selected data across multiple samples. You can customize which data you want to view for each plot, and you can modify the table at any time.

To create the Master Statistics Table:

1. In the Statistics Column Selector, enable the check boxes under the data items you want to view per plot. CFlow automatically adds columns to the Master Statistics Table.

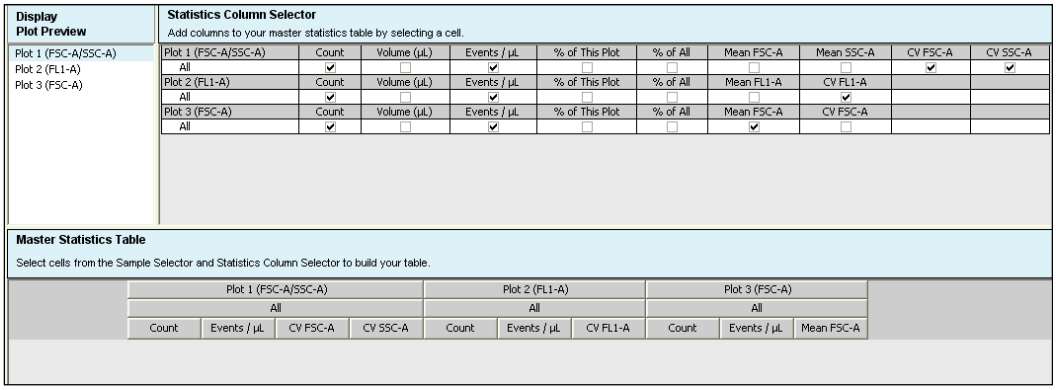

Display
Plot Preview
Plot 1 (FSC-A/SSC-A)
Plot 2 (FL1-A)
Plot 3 (FSC-A)
Statistics Column Selector
Add columns to your master statistics table by selecting a cell.
Plot 1 (FSC-A/SSC-A) Count Volume (µL) Events / µL % of This Plot % of All Mean FSC-A Mean SSC-A CV FSC-A CV SSC-A
All
Plot 2 (FL1-A) Count Volume (µL) Events / µL % of This Plot % of All Mean FL1-A CV FL1-A
All
Plot 3 (FSC-A) Count Volume (µL) Events / µL % of This Plot % of All Mean FSC-A CV FSC-A
All
Master Statistics Table
Select cells from the Sample Selector and Statistics Column Selector to build your table.
Plot 1 (FSC-A/SSC-A) Plot 2 (FL1-A) Plot 3 (FSC-A)
All All All
Count Events / µL CV FSC-A CV SSC-A Count Events / µL CV FL1-A Count Events / µL Mean FSC-A

Figure 5-2. Creating Master Statistics Table: Adding Plots

2. In the Sample Selector list, enable the check box of each sample you want to view. CFlow automatically adds rows of samples to the Master Statistics Table and displays the sample data.

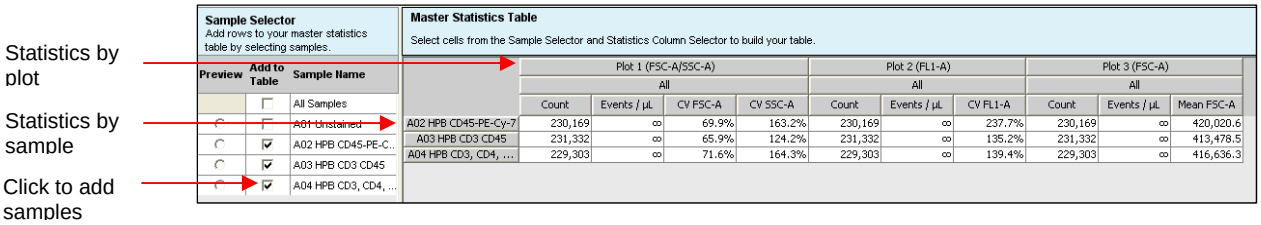

Statistics by plot
Statistics by sample
Click to add samples
Sample Selector
Add rows to your master statistics table by selecting samples.
Preview
Add to Table
Sample Name
All Samples
A01 Unstained
A02 HPB CD45-PE-C..
A03 HPB CD3 CD45
A04 HPB CD3, CD4, ...
Master Statistics Table
Select cells from the Sample Selector and Statistics Column Selector to build your table.
Plot 1 (FSC-A/SSC-A)
Plot 2 (FL1-A)
Plot 3 (FSC-A)
All
Count
Events / µL
CV FSC-A
CV SSC-A
CV FL1-A
Mean FSC-A
A02 HPB CD45-PE-Cy-7 230,169 ∞ 69.9% 163.2% 230,169 ∞ 237.7% 230,169 ∞ 420,020.6
A03 HPB CD3 CD45 231,332 ∞ 65.9% 124.2% 231,332 ∞ 135.2% 231,332 ∞ 413,478.5
A04 HPB CD3, CD4, ... 229,303 ∞ 71.6% 164.3% 229,303 ∞ 139.4% 229,303 ∞ 416,636.3

Figure 5-3. Creating Master Statistics Table: Adding Samples

5.3 Previewing a Plot in the *Statistics* Tab

To preview a plot in the *Statistics* tab:

1. In the Display Plot Preview list, click on the plot you want to preview.

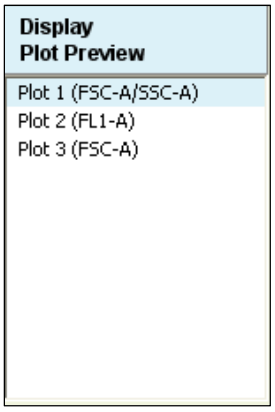


Figure 5-4. Plot Preview

2. In the Sample Selector list, select the radio button of a sample. CFlow displays the sample's data in the plot.

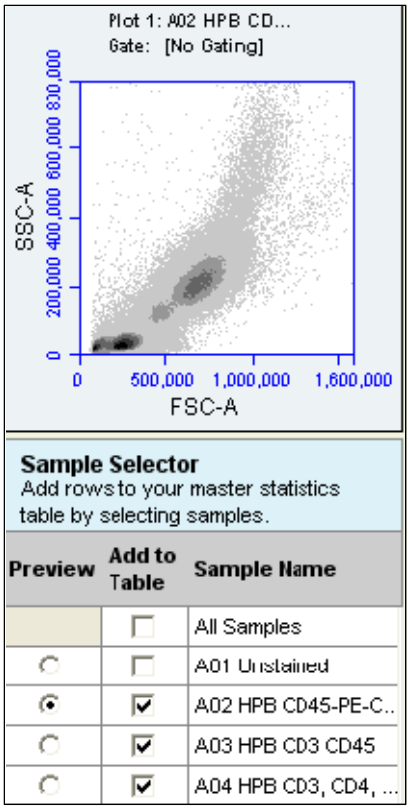


Figure 5-5. Sample Selector List and Plot

The plot preview is available for viewing only. You can modify the zoom level and other plot settings in the *Analyze* or *Collect* tab.

5.4 Copying Data into Other Applications

You can copy and paste data from the Master Statistics Table into most Microsoft Office compatible applications.

To copy data:

1. Use the mouse to highlight the fields you want to copy.
2. Press **Ctrl+C** to copy the data.
3. In the Microsoft application, press **Ctrl+V** to paste the data.

6 MAINTAINING THE C6

To ensure that your C6 Flow Cytometer maintains optimal performance, follow the routines in this chapter on a regular basis. See the *C6 Flow Cytometer Instrument Manual* for mechanical maintenance procedures (such as replacing tubing).

6.1 Cleaning the SIP

Run the backflush cycle to clean the SIP and remove clogs at the base of the SIP.

To perform a backflush:

1. Place a blotter or empty sample tube under the SIP to catch dripping fluid.
2. Click on the *Backflush* button to clear any residue from the SIP.
3. When the backflush is completed, remove the tube from the SIP.

6.2 Cleaning the Flow Cell

Clean the flow cell as a part of regular maintenance or to correct performance issues of the cytometer.

6.2.1 Running the Unclog Cycle

The unclog cycle purges the flow cell of debris.

To purge the flow cell:

1. Remove the sample tube from the SIP and place a blotter or empty sample tube under the SIP to catch dripping fluid.
2. Do one of the following:
   - Click on the *Unclog* button in the *Collect* tab.
   - Select *Instrument > Run Unclog Cycle.*

6.2.2 Running an Extended Clean of the Flow Cell

You can clean the flow cell for an extended time. During extended flow cell cleaning, the flow cell fills completely with cleaning solution from the sample tube on the SIP. This cycle automatically shuts down the C6 with cleaning solution in the flow cell, allowing the flow cell to soak.

To run the extended clean of the flow cell:

1. Place a tube with at least 500 µL of cleaning solution on the SIP.

   CAUTION: Never run the Extended Clean of the Flow Cell cycle without a tube containing at least 500 µL of fluid.
2. Select *Instrument > Extended clean of flow cell*.
3. After the C6 is shut down, leave the cytometer off for at least 30 minutes (or longer, for a more thorough cleaning).

4. Restart the cytometer. The C6 performs a longer fluidics startup cycle and CFlow displays the message *Extra startup time needed due to cleaning or improper shutdown*. This longer cycle purges cleaning solution from the flow cell.
5. Operate the C6 as usual when startup is complete.

6.3 Cleaning the Fluidics Lines

The cleaning fluid cycle pulls cleaning solution from the cleaner tank and runs it through the fluidic lines. After filling the system with cleaning solution, the cleaning fluid cycle purges the cytometer with fresh sheath fluid and performs a backflush. This cycle takes about five minutes.

To run a cleaning fluid cycle:

1. Place a tube of cleaning solution on the SIP.
2. Select *Instrument* > *Run cleaning fluid cycle*.

6.4 Decontaminating the Fluidics System

CFlow decontaminates the fluidics system of biohazardous materials every time you shut down the C6. You can also manually run the decontamination fluid cycle at any time. Decontamination takes about 13 minutes.

To manually decontaminate the fluidics:

1. Place a tube of water on the SIP.
2. Select *Instrument* > *Run decontamination fluid cycle*. The C6 pulls decontamination fluid from the decontamination bottle then pulls sheath fluid from the sheath fluid bottle.

6.5 Calibrating the Fluidics System for Precise Volume Measurements

If you validate performance with the Accuri Volumetric Validation Beads (QA-120) and validation fails, you can recalibrate the C6 Cytometer.

Do the following on a regular basis to ensure that the C6 continues to be correctly calibrated:

- Replace any peristaltic pump tubing that is at least two months old.
- Run samples at medium or fast speeds.
- Use the same sample volume/fluid height in the tube during calibration as you will use during your experiment.

To calibrate the C6:

1. Fill a tube with 500 µL of sheath fluid.

   NOTE: Use the same kind of tube that you will use during the experiment.
2. Place the tube on the SIP.
3. In CFlow, select *Instrument* > *Calibrate fluidics* to begin calibration. The calibration takes about five minutes.

If calibration fails, the C6 automatically reverts to the factory calibration settings and operates normally. However, the volumes recorded will not be optimized for the new sample volume. If more precise measurement is essential, try calibrating again.

7 ADJUSTING PEAK POSITION WITH VIRTUALGAIN®

In certain instances, a particular peak should have the same position across different samples or be located at a specific channel number, regardless of the staining. Instruments that have voltage and amp gain controls allow you to adjust peak position from sample to sample. CFlow uses VirtualGain instead of these controls.

VirtualGain is a software module that mimics voltage and amp gain adjustments to reposition data on the axis after the data has been collected. VirtualGain makes gross adjustments (approximate visual shifts of the data) of histogram plots. It is strictly an analysis tool and should not be used while collecting data.

For example, in Figure 7-1 the negative peaks in the control sample and in sample 1 fall in similar channels (mean value = 28.2 and 29.7, respectively). However, the negative population in sample 2 is farther to the right (mean value = 73.4). You can use VirtualGain to align the negative peak of sample 2 with the control sample.

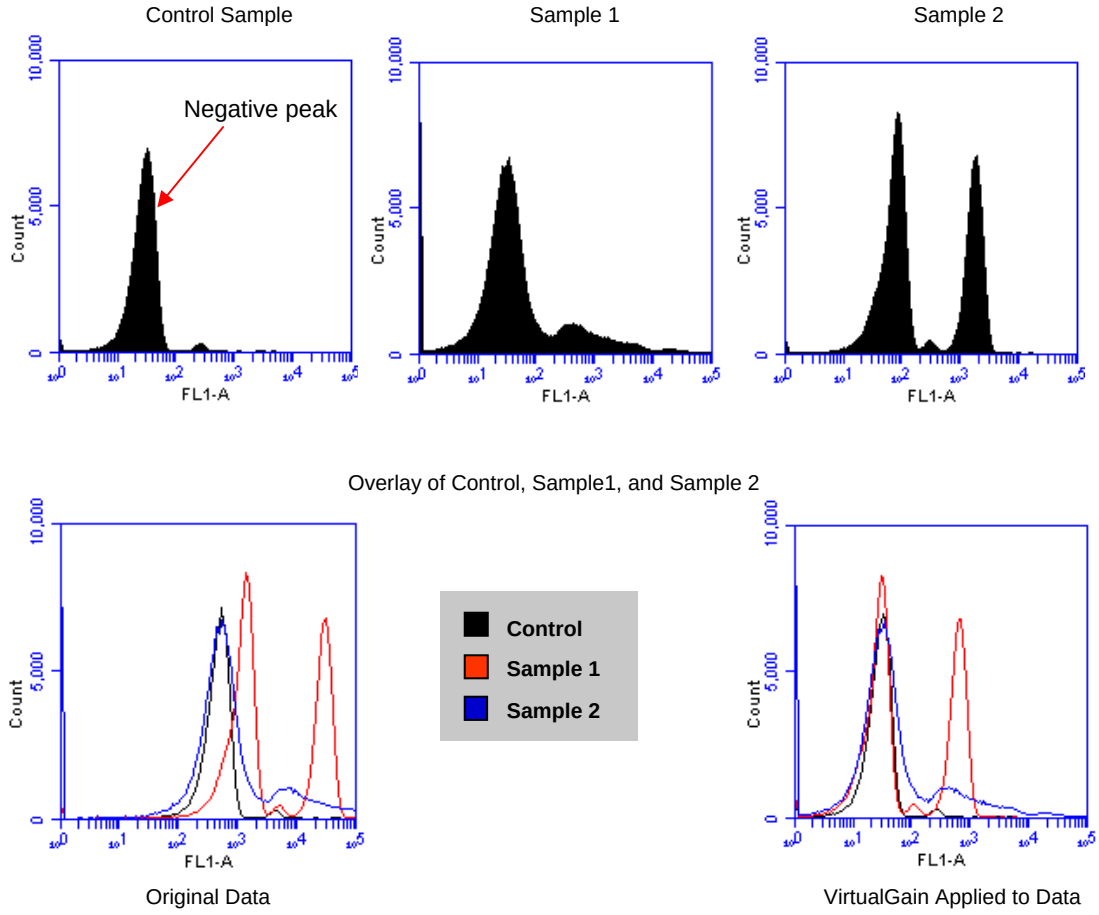


Figure 7-1. Before and After Applying VirtualGain

7.1 Applying VirtualGain

Only apply VirtualGain on a histogram plot, and only on one parameter at a time. After VirtualGain is applied, you can view data in any type of plot and toggle VirtualGain on and off.

VirtualGain is only applied to the displayed data and does not alter FCS data. The adjustment is recorded only in the CFlow file.

To apply VirtualGain:

1. In the *Analyze* tab, do one of the following:
   - Recreate the histogram to which you want to apply VirtualGain (see section 4.2.3 for details).
   - Copy plots from the *Collect* tab (see section 4.2.1 for details).

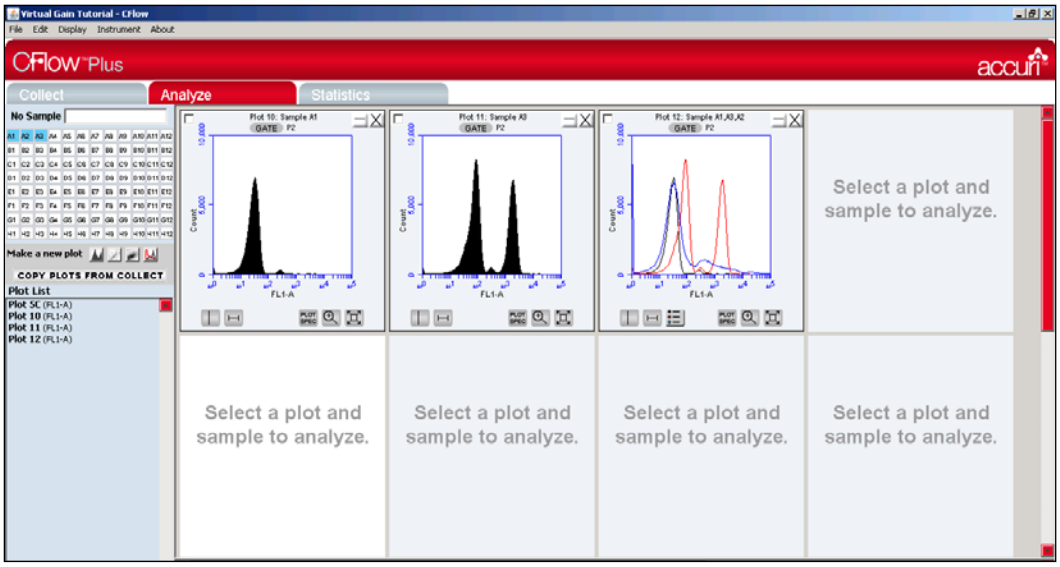


Figure 7-2. Setting Up Histograms for VirtualGain

2. Apply the appropriate gating to the plots in the *Analyze* tab.
3. Do one of the following:
   - Select a histogram plot from the sample to which the other samples will be aligned. This sample is the *standard sample.*
   - Select an empty well if you want to align data to a *specific channel* instead of a collected sample.
4. Click on the x-axis label on the standard sample and select *VirtualGain* from the pop-up Parameter List.

5. In the VirtualGain dialog box, do one of the following:
   - Move the peak definition marker (vertical line) in the Standard Sample plot to the center of the peak that will be the reference point. Other samples will be aligned to this position.
   - If you selected an empty well in step 3, move the peak definition marker (vertical line) to the channel that you want to assign as the reference point.

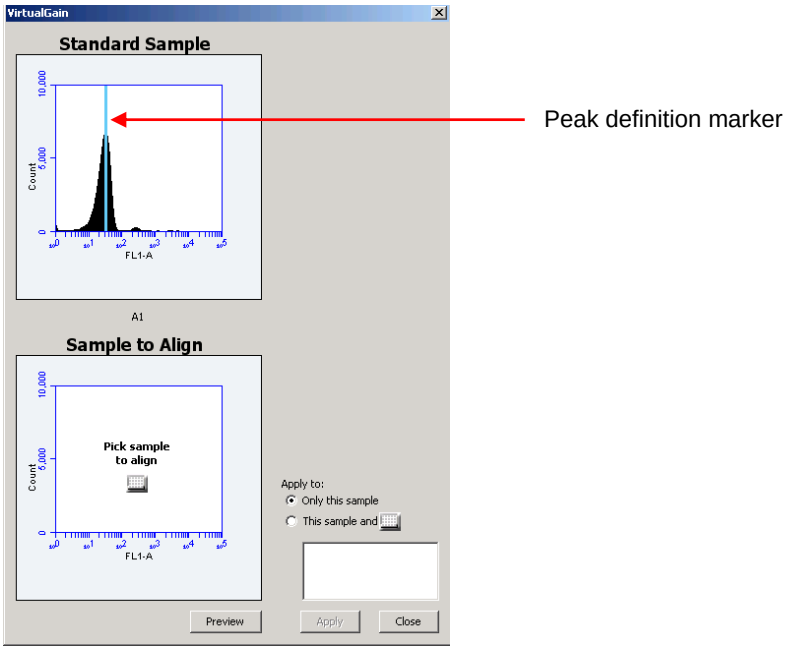


Figure 7-3. Aligning Plots

6. If needed, use the Zoom Tools in the *Analyze* tab to change the zoom level in the VirtualGain dialog box.
7. Click on the small sample grid icon in the center of the Sample to Align plot.

8. Open the sample to be aligned by clicking on the corresponding blue well in the pop-up sample grid (the gray well indicates the standard sample currently selected). Ensure that this plot has been zoomed to the required level before setting VirtualGain.

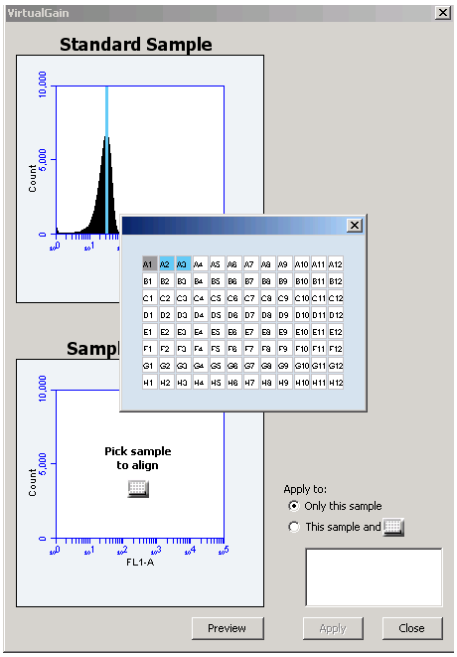


Figure 7-4. Pick the Sample to Align

9. Move the peak definition marker in the Sample to Align plot to the center of the peak that you want to align (Figure 7-5).

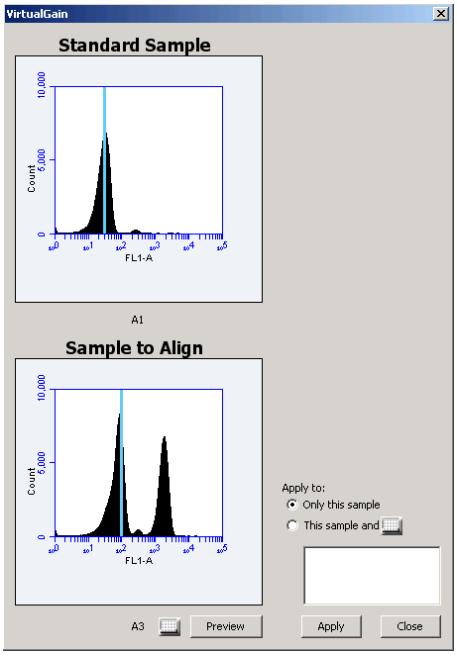


Figure 7-5. Move the Peak Definition Marker

10. Click on the *Preview* button to view the aligned sample with VirtualGain applied. CFlow aligns the peak of interest in both plots.
11. Repeat steps 9-10 to make additional adjustments, if needed.
12. To align additional samples exactly as the first aligned sample, select the *This sample and* radio button and click on the well(s) in the pop-up sample grid that you want to include. If the other samples need a different amount of VirtualGain, set VirtualGain separately for each one.

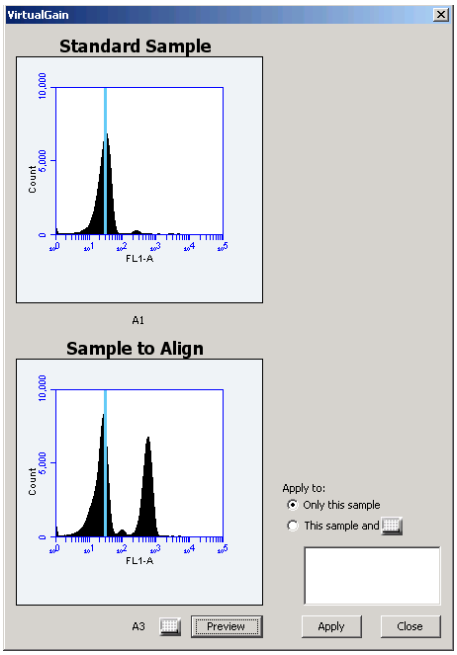


Figure 7-6. Align Additional Samples

13. Click on the *Apply* button to apply VirtualGain to the data. CFlow displays a black asterisk under the Sample to Align plot to indicate that VirtualGain has been applied to the specified parameter for that sample.

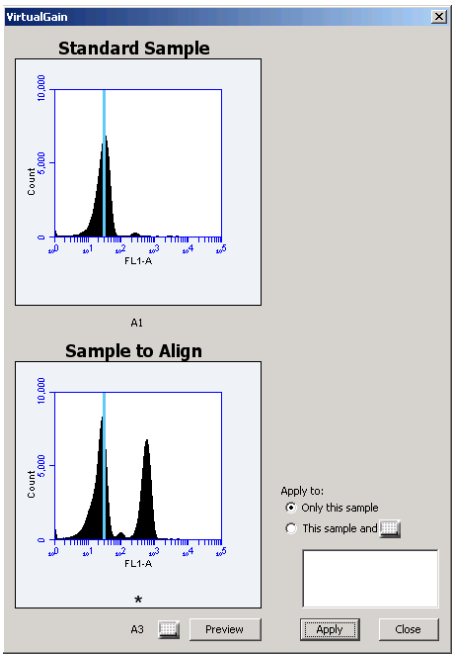


Figure 7-7. Black Asterisk Identifier

14. Click on the *Close* button to close the VirtualGain dialog box.

7.2 Viewing VirtualGain

When VirtualGain is applied to a sample, CFlow displays a black asterisk under the parameter label in the associated plot.

The asterisk is color-coded:

- Black—VirtualGain has been applied.
- Gray—CFlow is currently displaying the original data.

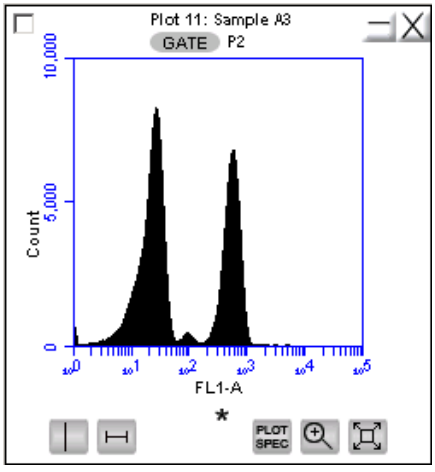


Figure 7-8. Plot with Black Asterisk

Overlays automatically display VirtualGain when it is applied (as shown in the following figure). The asterisk is not displayed in overlays when VirtualGain is applied to some or all of the samples in the overlay.

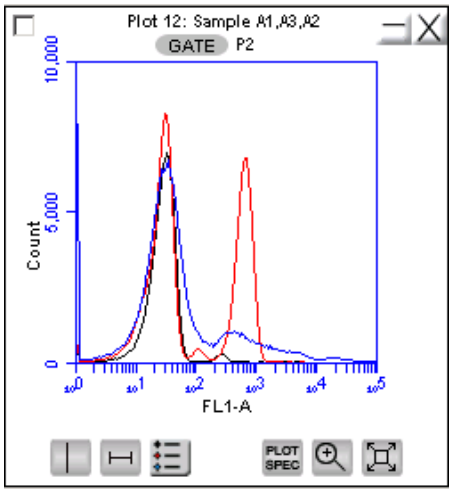


Figure 7-9. VirtualGain Applied in an Overlay Histogram

To toggle between views with VirtualGain applied and not applied:

1. Click on the asterisk in the plot.

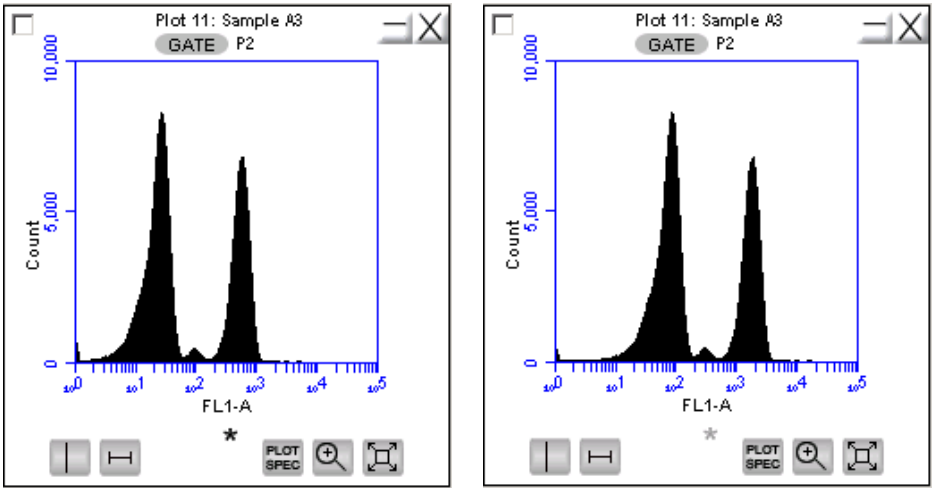


Figure 7-10. Toggle between VirtualGain Applied (Left) and Not Applied (Right)

7.3 Removing VirtualGain

To permanently remove VirtualGain from every parameter in every sample in the CFlow file:

1. Select *Display* > *Remove All VirtualGain*.

CAUTION: You cannot undo this action.

APPENDIX A CFLOW MENU QUICK REFERENCE

The following table provides a description of all the menu options in CFlow.

Table A-1. CFlow Menus

Menu Item	*Menu pick*	*Description*
File	Open CFlow File or Template	Opens a previously saved CFlow file or CFlow template. Only one CFlow workspace can be open at a time.
	New CFlow File	Opens a new, blank CFlow workspace. Replaces any previously open workspace.
	Save	Saves the open CFlow workspace under the current name. If the file has not already been named, CFlow prompts you to name the file.
	Save CFlow File as	Saves the open CFlow workspace under a new name.
	Save CFlow Template as	Creates a template from the currently open CFlow workspace. All markers, regions, gates, parameter names, and sample names are saved without any data points.
	Auto-save Settings	Allows you to enable or disable the auto-save feature.
	Import FCS File	Imports an FCS file previously exported from another CFlow file to the currently open workspace. Only FCS files created on a C6 Flow Cytometer can be imported into CFlow.
	Export FCS File	Exports and saves the currently selected data well as an FCS 3.0 file to a specified folder. Exported files are compatible with off-line analysis programs such as FCS Express, FlowJo, and WinList.
	Export ALL Samples as FCS	Saves all of the data wells as individual FCS 3.0 files in the folder `CFlow-FCS Exports` on the compouter desktop. Exported files are compatible with off-line analysis programs such as FCS Express, FlowJo, and WinList.
	Export ALL Samples to Third Party	Exports and saves all data wells as individual FCS 3.0 files that enable autoscaling in third party applications (such as FlowJo).
	Export Plot Data as CSV	Saves an individual file in .csv format for further analysis in spreadsheet programs. All data for every event in the selected plot is exported. See APPENDIX G for an example .csv file.
	Print Selected Items	Prints selected plots and associated statistics.
	Quit	Quits CFlow and closes the application.
Edit	Undo	Undoes the last action that was performed in CFlow. Not all actions are undoable.
	Redo	Reverses an *Undo* action.
	Copy	Copies a marker or region from a plot, or statistics from the tables in CFlow.

Menu Item	*Menu pick*	*Description*
Edit	Paste	Pastes copied markers and regions into new plots.
	Rename Parameters	Allows you to rename individual parameters in either the current sample or all samples at the same time.
Display	Events Display Settings	Opens a dialog that allows you to change the number of events displayed in all plots.
	Remove All VirtualGain	Removes all VirtualGain settings from the entire CFlow workspace. VirtualGain settings are removed from all wells of data.
	Hide/Show Median Statistics	Hides/shows the median statistics in the Statistics Table.
Instrument	Set threshold	Opens the Threshold dialog box for selecting the trigger channel, setting the primary threshold value, and setting an optional secondary threshold.
	Set Compensation	Opens the Compensation Settings dialog box for subtracting fluorescence spillover.
	Run Cleaning Fluid Cycle	Runs a cleaning fluid cycle.
	Run Decontamination Fluid Cycle	Allows you to manually run the decontamination fluid cycle.
	Run Unclog Cycle	Runs the unclog cycle to clean the flow cell.
	Run Backflush Cycle	Runs the backflush cycle to clean the SIP and remove clogs at the base of the SIP.
	Extended Clean of Flow Cell	Cleans the flow cell for an extended time.
	Calibrate Fluidics	Initiates fluidics calibration to ensure that the C6 provides accurate measurement of the volume aspirated from samples.
	Update Firmware	Updates the C6 firmware. Use only when directed by Accuri Cytometers to upgrade the C6 firmware with an official firmware release.
	Remote Control	Optional feature that allows control of the C6 from a remote location.
About	About CFlow	Opens a dialog box that displays the version of CFlow and Accuri Technical Support contact information.
	Technical Support Information	Opens a dialog box that displays information about CFlow and the C6. Each time an activation key is used to install a new CFlow component, the dialog box is updated to reflect the change.
	Users	Optional feature that allows you to add, delete, or modify user accounts for usage tracking.
	Get C6 Internal Log	Not currently available.

APPENDIX B ADVANCED FLUIDICS SETTINGS

Advanced users can customize the fluidics rate and core size for collecting samples.

To customize the fluidics rate:

1. Select the *Custom* radio button in the Fluidics section of the *Collect* tab.

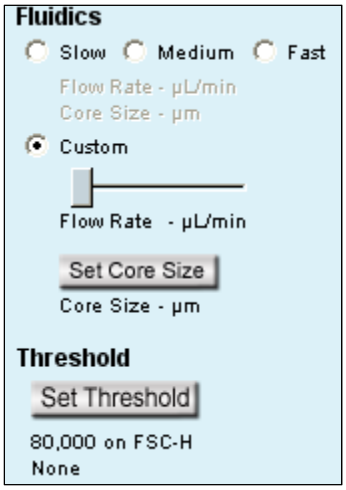


Figure B-1. *Custom* Radio Button

2. Move the *Custom* slider to the rate you want to set.

To customize the sample core size:

1. Click on the *Set Core Size* button in the Fluidics section of the *Collect* tab.
2. Move the slider to the core size you want to set.

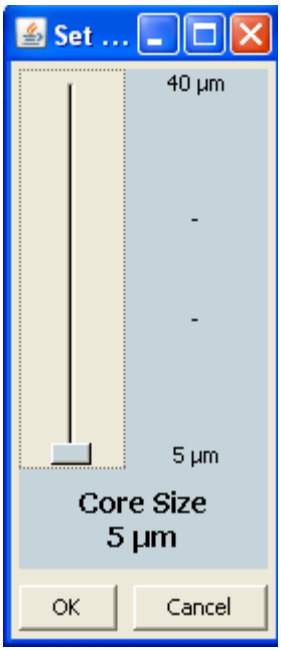


Figure B-2. Set Core Size

NOTE: Certain core sizes are not possible at certain flow rates. CFlow does not allow you to set these combinations. You can use the following table to determine allowable combinations.

Table B-1. Core Size/Flow Rate Combinations

Core Size	*Min Flow Rate*	*Max Flow Rate*
5	10	11
6	10	16
7	10	22
8	10	29
9	10	36
10	10	45
11	10	54
12	10	65
13	10	76
14	12	88
15	14	100
16	15	100
17	17	100
18	19	100
19	22	100
20	24	100
21	26	100
22	29	100
23	32	100
24	35	100
25	38	100
26	41	100
27	44	100
28	47	100
29	50	100
30	54	100
31	58	100
32	61	100
33	65	100
34	69	100
35	74	100
36	78	100
37	82	100
38	87	100
39	91	100
40	96	100

3. Click on the *OK* button to set the core size and close the slider.

APPENDIX C TRACKING USER ACTIVITY

User tracking allows laboratory administrators to track the activities of C6 operators by assigning a user name and password to each individual. Passwords are created and used in CFlow and are unrelated to any Windows passwords used on the host computer or network.

User Tracking is an optional upgrade for CFlow Plus and CFlow Sampler software and requires the use of CFlow version 227 or above.

C.1 Installing the User Tracking Module

To install user tracking on your CFlow computer:

1. Ensure CFlow Plus or CFlow Sampler version 227 or above is loaded on the computer.
2. Copy the User Tracking Activation Key from the installation CD or flash drive to the computer desktop.
3. Double-click on the User Tracking Installer icon on the desktop.

Figure C-1. User Tracking Installer Icon

4. In the installation wizard, choose the correct directory to install the Activation Key. The location depends on where CFlow Plus or CFlow Sampler has been installed. In most cases, the location is in one of two places:
   - C:\Program Files\CFlow\CFlow Plus\ActivationKeys
   - C:\Program Files\CFlow\CFlow Sampler\ActivationKeys

   If necessary, use the browse button ... to navigate to the correct folder.

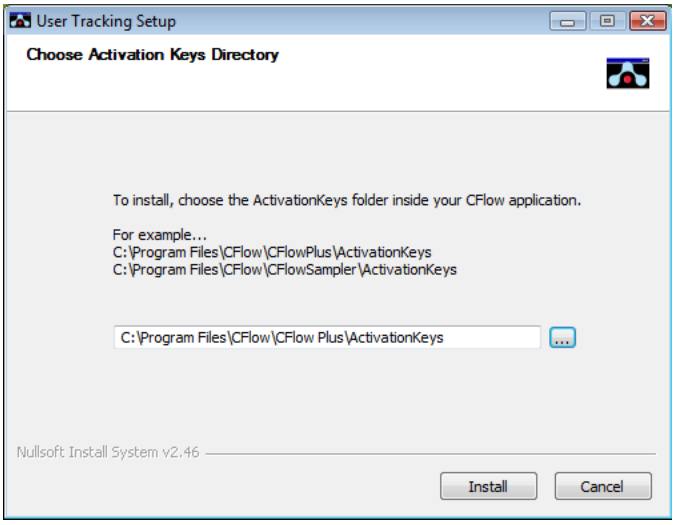


Figure C-2. Installation Dialog Box

5. Click on the *Install* button. CFlow displays a confirmation message after successful installation.

C.2 Using the Tracking Feature for the First Time

To use the tracking feature for the first time:

1. Open CFlow.
2. When prompted, type *admin* in the *Username* text box.

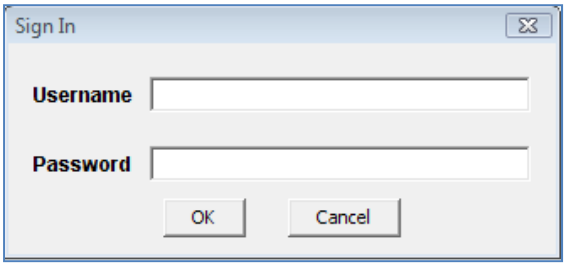


Figure C-3. Username and Password Dialog Box

3. Type *Admin* in the *Password* text box (case sensitive). You can change the Administrator password later.
4. Click on the *OK* button
5. Use CFlow as usual.

C.3 Adding, Deleting, and Modifying User Accounts

The administrator can add new users, delete existing users, and change the administrator password.

C.3.1 Adding User Accounts

To add new user accounts:

1. Sign in as the administrator.
2. Select *About* > *Users* in CFlow.

   NOTE: The *Users* menu option is only visible to the administrator.

3. In the Users dialog box, click on *Add New User.*

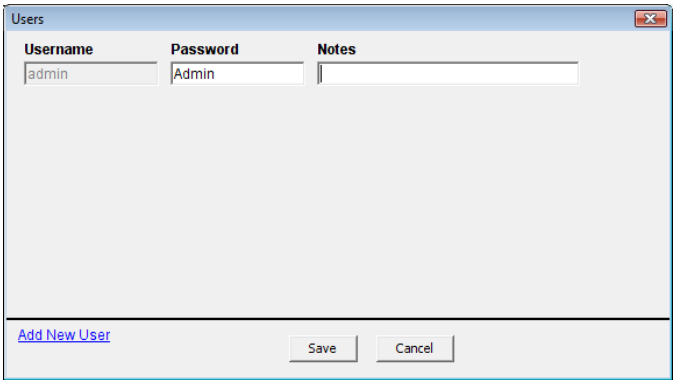


Figure C-4. The Users Information Box

4. Type the *Username* and *Password* for the user in the blank text boxes. Type any notes you want to add in the *Notes* text box (this information is only visible in the Users dialog box).

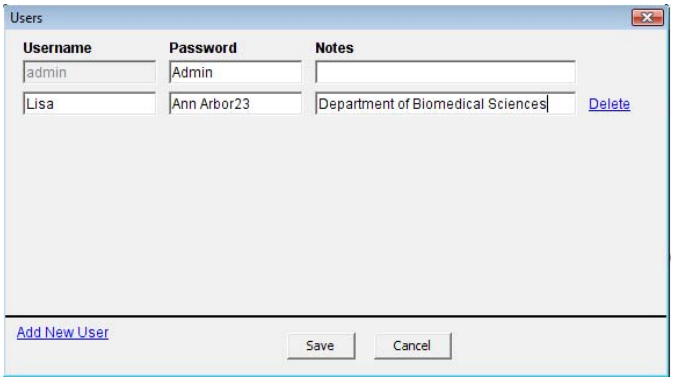


Figure C-5. Adding a New User

5. Click on the *Save* button to save your changes.

C.3.2 Deleting User Accounts

To delete a user account:

1. Select *About* > *Users* in CFlow.

2. Click on *Delete* next to the user account you want to remove.

   NOTE: You cannot delete the administrator's account.

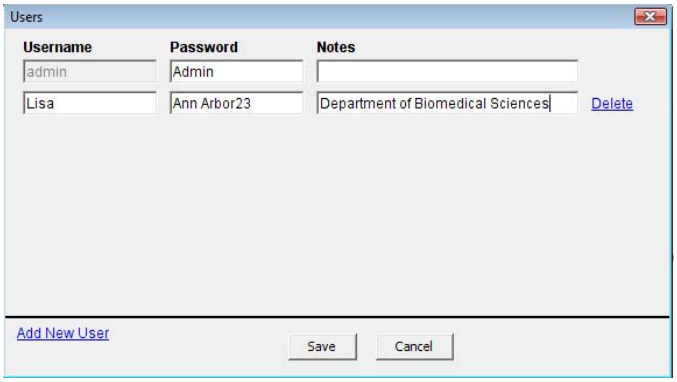


Figure C-6. Deleting a User

3. Click on the *Save* button to save your changes.

C.3.3 Changing a Password

The administrator can change a user's password at any time.

To change a user password:

1. Select *About* > *Users* in CFlow.
2. Delete the text in the *Password* field and type a new password.
3. Click on the *Save* button.

C.4 Signing In and Signing Out

Once tracking is set up, users must sign in for each session.

To sign in to CFlow:

1. Open CFlow.
2. When prompted, type your *Username* in the text box.

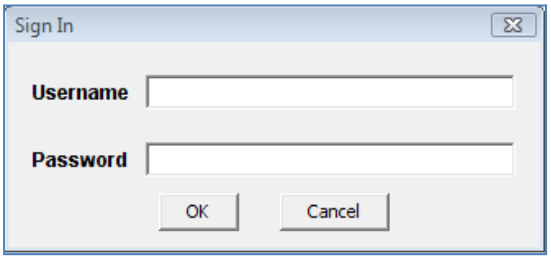


Figure C-7. Username and Password Dialog Box

3. Type your *Password* in the text box.
4. Click on the *OK* button
5. Use CFlow as usual.

Be sure you sign out of CFlow each time you end your session. If you do not sign out, CFlow will continue to log your time.

To sign out of CFlow:

1. In CFlow, select *File* > *Quit.*

C.5 Restoring a Forgotten Administrator Password

The password file is encrypted and contains all username and password information created in the User Logging feature. If you forget your Administrator password, you can delete the password file and recreate your Administrator account.

CAUTION: This procedure deletes *all* user names and passwords; you must manually recreate each account.

To restore the administrator password:

1. If CFlow is running, shut down CFlow.
2. Navigate to the CFlowSupportFiles folder (typically in the root directory of the CFlow computer).
3. Locate the Password file and delete the file.
4. Follow the procedures in sections C.2 and C.3.1.

C.6 Monitoring User Activity

Each time a user signs into or signs out of CFlow, an entry is made in the userUsage Log. The userUsage log is a text file that you can view in a text editor (such as Notepad) or copy into Microsoft Excel as a comma delimited file.

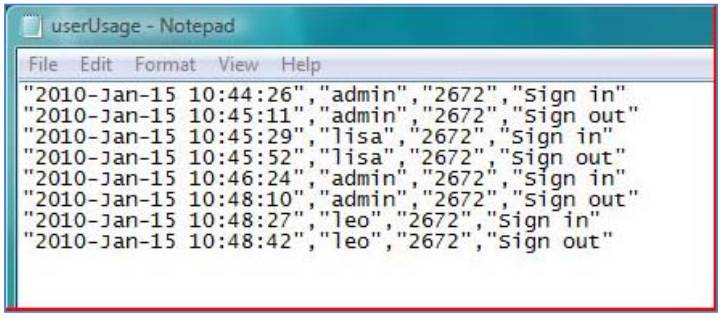


Figure C-8. userUsage Log

The userUsage Log contains the following information:

- Date of a sign in/sign out
- Time of a sign in/sign out
- Username of the operator
- Serial number of the C6
- Type of activity (sign in or sign out)

To view the userUsage log:

1. Navigate to the CFlowSupportFiles folder (typically in the root directory of the CFlow computer) and open the folder.

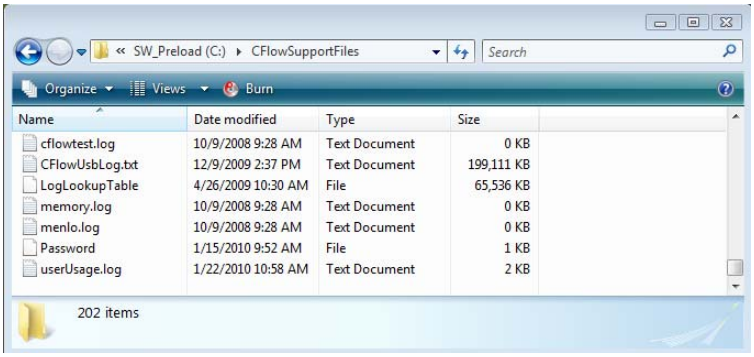

SW_Preload (C:) ▸ CFlowSupportFiles
Search
Organize
Views
Burn
Name
Date modified
Type
Size
cflowtest.log 10/9/2008 9:28 AM Text Document 0 KB
CFlowUsbLog.txt 12/9/2009 2:37 PM Text Document 199,111 KB
LogLookupTable 4/26/2009 10:30 AM File 65,536 KB
memory.log 10/9/2008 9:28 AM Text Document 0 KB
menlo.log 10/9/2008 9:28 AM Text Document 0 KB
Password 1/15/2010 9:52 AM File 1 KB
userUsage.log 1/22/2010 10:58 AM Text Document 2 KB
202 items

Figure C-9. CFlowSupportFiles Folder

2. Open the userUsage.log file.

APPENDIX D CFLOW ANALYSIS SOFTWARE

The optional CFlow Analysis software allows you to analyze CFlow files on a computer that does not have a connection to the C6 Flow Cytometer. CFlow Analysis is available in two forms:

- CF-20—CFlow Plus Analysis Software for PC or Mac
- CF-30—CFlow Sampler Analysis Software for PC or Mac

CFlow Analysis has all the functionality of CFlow, but the instrument control functions in the *Instrument* menu are disabled (except *Set Threshold* and *Set Compensation*). CFlow Analysis software is not intended for controlling the C6 for data acquisition.

MINIMUM SYSTEM REQUIREMENTS

- PC version:
  - Intel Core Duo processor, 2.8GHz
  - 2GB RAM
  - CD-ROM Drive
  - Windows® XP (Service Pack 2) or Windows Vista (recommended)
  - Language selection: US or UK English
  - 5GB hard disk space
  - Display resolution 1152 x 864 or higher
- Mac version:
  - Intel or PowerPC processor, 1.66 GHz (Intel processor recommended)
  - 1GB RAM
  - CD-ROM Drive
  - Mac OS X software
  - Language selection: US or UK English
  - 5GB hard disk space
  - Display resolution 1152 x 864 or higher

SOFTWARE OPERATION

See the relevant chapters in this guide for operational information.

APPENDIX E SELECTABLE LASERS

The detectors and lasers of the standard C6 Flow Cytometer System operate in a predefined configuration: detectors FL1, FL2, and FL3 read blue laser-excited fluorescence emissions and detector FL4 reads red laser-excited emissions. This configuration is referred to as *3 blue 1 red*.

The Selectable Lasers Module (CM-50) allows you to operate the C6 in two other configurations to significantly expand the fluorochrome combinations that can be analyzed (see section E.4 for examples):

- *2 blue 2 red:*
  - FL1 and FL2 read blue laser-excited emissions
  - FL3 and FL4 read red laser-excited emissions
- *4 blue:* All 4 detectors read blue laser-excited emissions

Components Supplied:

- Selectable Lasers Activation Key (software)
- Three optical filters:
  - 780/60 (CP-176)
  - 610/20 (CP-174)
  - 630/30 (CP-180)

NOTE: The Selectable Lasers upgrade requires prior installation of CFlow® Plus or CFlow Sampler.

E.1 Installing the Selectable Lasers Module

To install the Selectable Lasers module:

1. Verfiy that CFlow Plus or CFlow Sampler version 227.4 or above is loaded on the computer. The Selectable Lasers Module can also be installed on any computer where CFlow Analysis (version 227.4 or higher) is installed.
2. Copy the Selectable Lasers Activation Key from the installation CD (or flash drive) to the desktop.
3. Double-click on Selectable Lasers Activation Key icon.

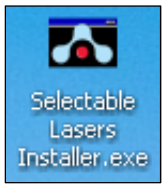


Figure E-1. Selectable Lasers Installer

4. In the installation wizard, choose the correct directory to install the Activation Key. The location depends on where CFlow Plus or CFlow Sampler has been installed. In most cases, the location will be in one of the following places:
   - C:\Program Files\CFlow\CFlow Plus\ActivationKeys
   - C:\Program Files\CFlow\CFlow Sampler\ActivationKeys

   NOTE: If you have CFlow Plus and CFlow Sampler on the same computer, install the Selectable Lasers Activation Key twice: once in the CFlow Plus directory, and again in the CFlow Sampler directory.

   Click on the browse button [...] to navigate to the correct location.

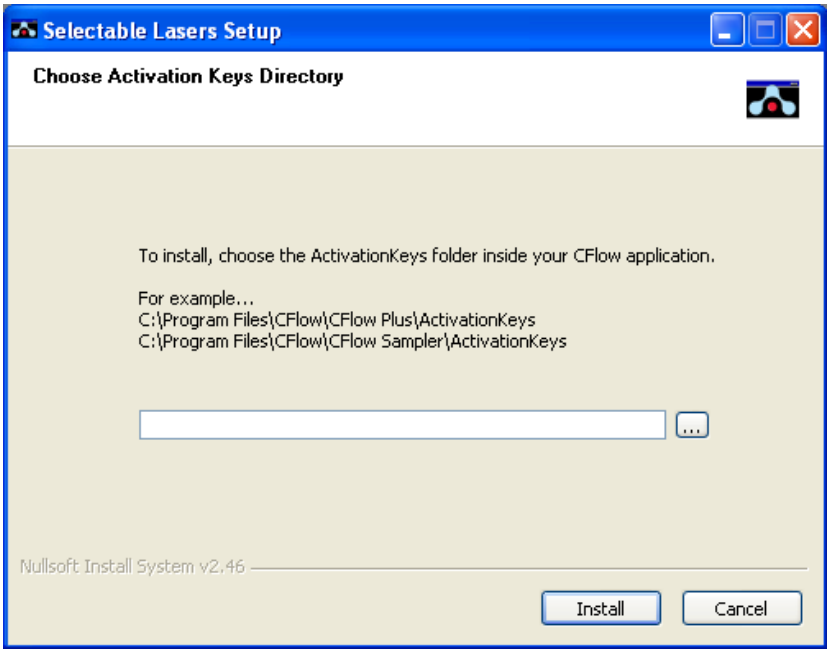


Figure E-2. Install Wizard: Choose Activation Keys Directory

5. Click on the *Install* button.

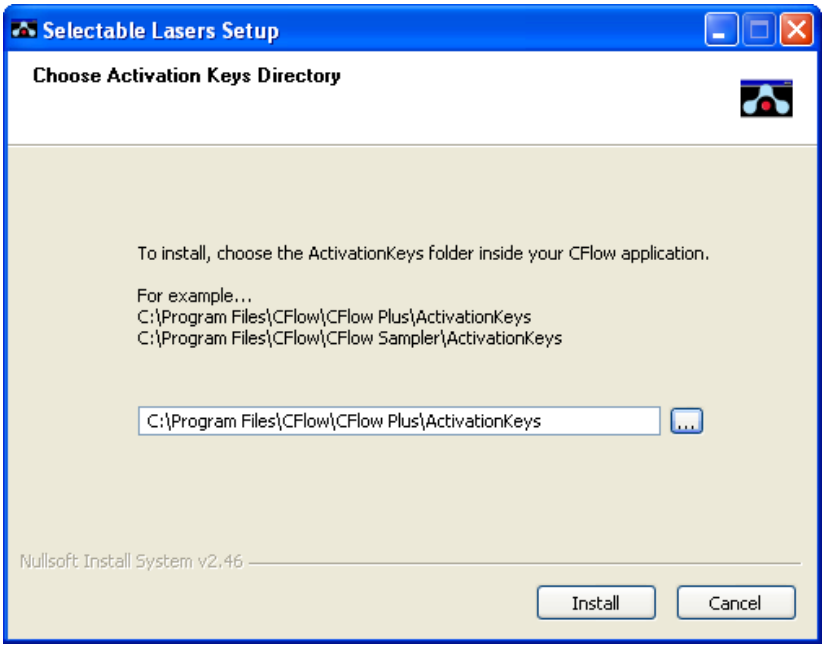


Figure E-3. Install Wizard: Install the Module

6. After installation, click on the *Close* button.

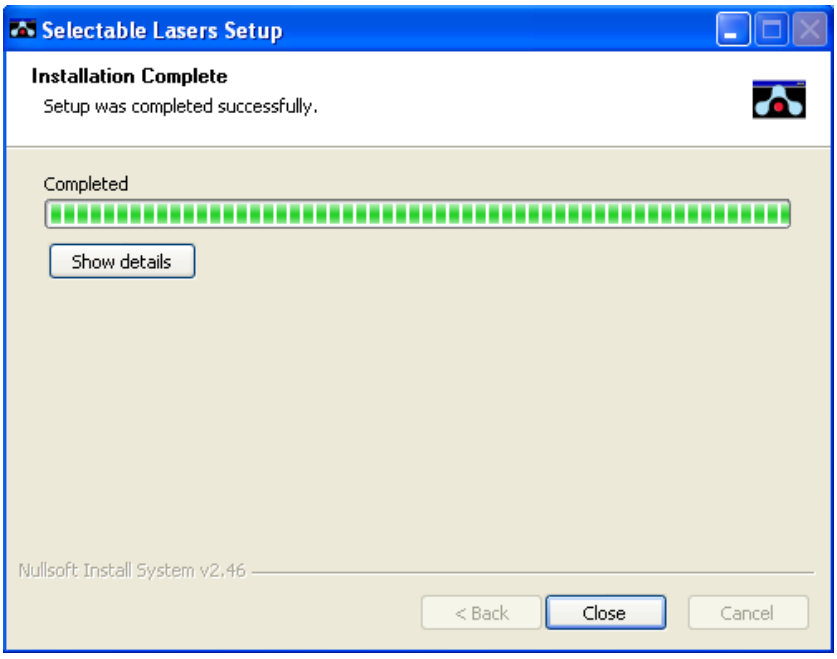


Figure E-4. Install Wizard: Setup Completed Successfully

7. Start CFlow. The Selectable Lasers controls are displayed next to the CFlow traffic light message.

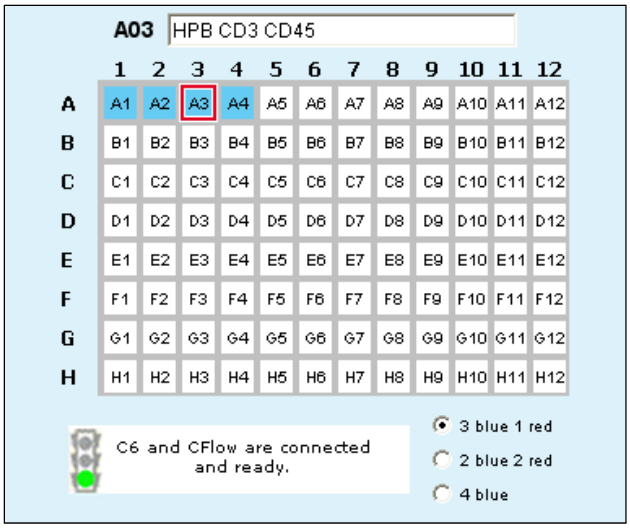

A03
HPB CD3 CD45
1 2 3 4 5 6 7 8 9 10 11 12
A A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12
B B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12
C C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12
D D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12
E E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12
F F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12
G G1 G2 G3 G4 G5 G6 G7 G8 G9 G10 G11 G12
H H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H12
C6 and CFlow are connected and ready.
3 blue 1 red
2 blue 2 red
4 blue

Figure E-5. Selectable Laser Controls in the CFlow Workspace

8. Depending on the laser configuration you are going to use, you may need to replace the standard optical filters with one or more of the filters that are supplied with the Selectable Lasers software. See section E.4 for details.

E.2 Validating Proper Function After Installation

After installing the Selectable Lasers Module, verify that the module is operating properly. To verify the Selectable Lasers function:

1. Leave the standard optical filters in place.
2. With CFlow open and the C6 powered on, select the *3 blue 1 red* laser configuration.

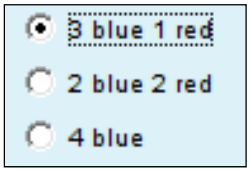


Figure E-6. Selectable Laser Options: *3 Blue 1 Red*

3. Set the run limit to 30,000 events in all and set the fluidics rate to slow.
4. Collect data files for the 8- and 6-Peak Validation Beads (Accuri Part #QA-100, QA-110).
5. Confirm that the C6 is operating within specification (see the *CFlow User Guide* for details).

6. Select the *2 blue 2 red* option and collect a data file using the 6-Peak Validation Beads.

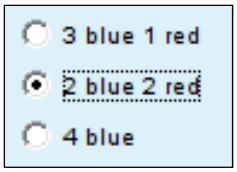


Figure E-7. Selectable Laser Options: *2 Blue 2 Red*

7. Select the *4 blue* option and collect a data file using the 8-Peak Validation Beads.

Figure E-8. Selectable Laser Options: *4 Blue*

8. Confirm proper operation of the *2 blue 2 red* configuration by comparing the 6-peak bead distributions for FL3 and FL4 to those obtained with the standard *3 blue 1 red* configuration. Results for the *2 blue 2 red* selection should show 6 peaks for both FL3 and FL4.

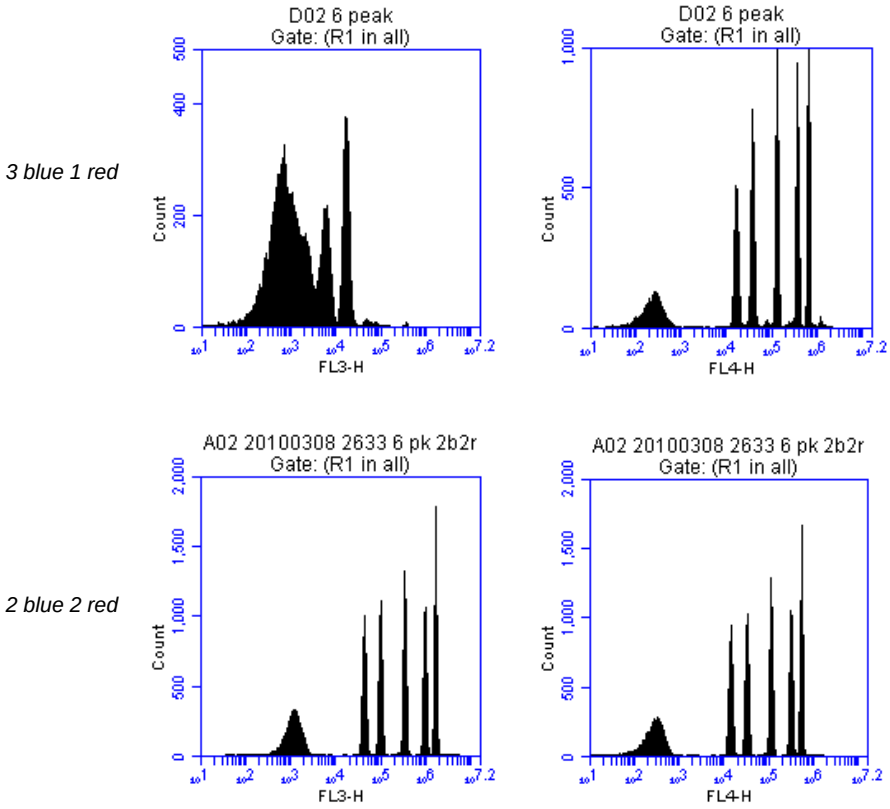


Figure E-9. Evaluation of 6-Peak Validation Beads for the *2 blue 2 red* configuration

9. Confirm proper operation of the *4 blue* configuration by comparing the 8-peak distributions for FL3 and FL4 to those obtained with the standard *3 blue 1 red* configuration. Results for the *4 blue* selection should show similar peak profiles for FL3 and FL4.

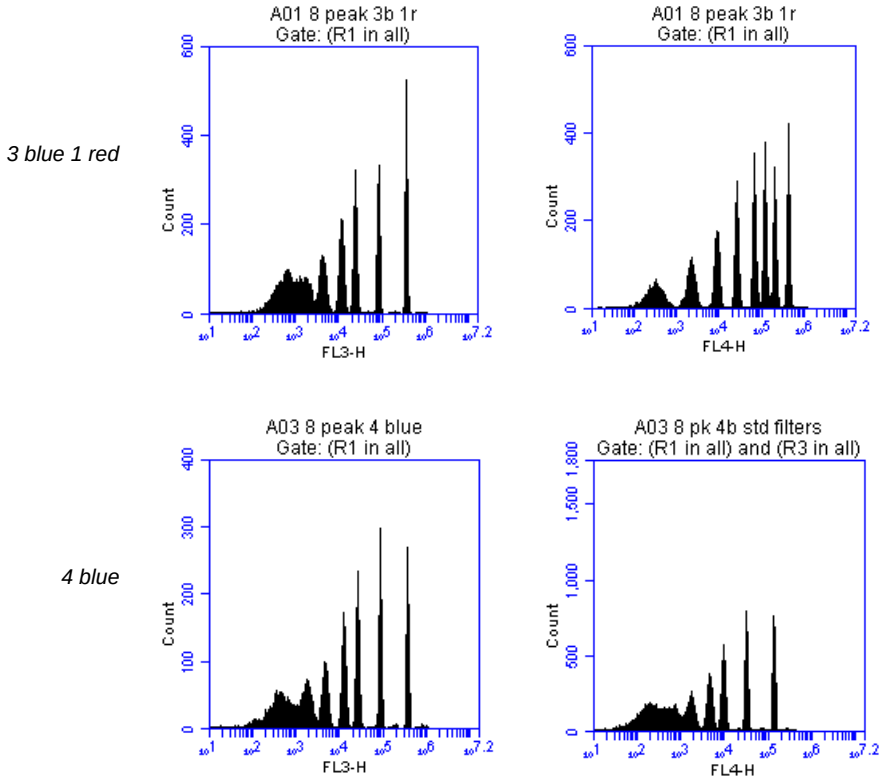


Figure E-10. Evaluation of 8-Peak Validation Beads for the *4 Blue* Configuration

E.3 Annotating Selected Laser Configuration

The selectable laser radio buttons show the *most recently* selected configuration only; they do not change if you select another data well with a different configuration. It is recommended to annotate your data wells in the naming field of CFlow to indicate the laser configuration used during data collection of each well, especially when not using the default *3 blue 1 red* option. (For example, name a sample “HPB 4b” to indicate that the *4 blue* option was selected during data collection.)

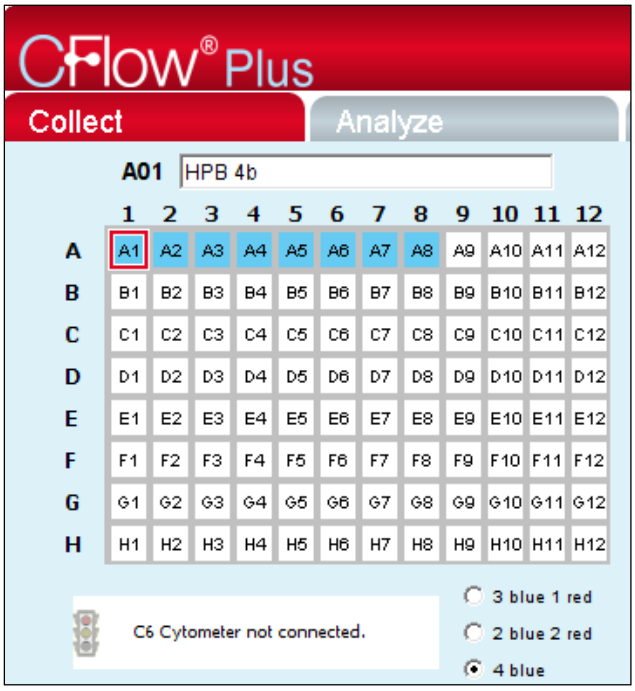

CFlow® Plus
Collect
Analyze
A01
HPB 4b
1 2 3 4 5 6 7 8 9 10 11 12
A A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12
B B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12
C C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12
D D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12
E E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12
F F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12
G G1 G2 G3 G4 G5 G6 G7 G8 G9 G10 G11 G12
H H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H12
C6 Cytometer not connected.
3 blue 1 red
2 blue 2 red
4 blue

Figure E-11. Renamed Data Well to Indicate *4* Blue Configuration

Laser configurations are *not saved* in the CFlow data file or with a CFlow template. Any previously saved CFlow file will default back to the *3 blue 1 red* option when it is opened.

The last laser configuration used to collect a given well of data is written in the FCS file header using the custom keyword **#LASERCONFIGURATION.** FCS file headers can be viewed by opening an exported FCS file in a text editor (such as Microsoft® Notepad).

E.4 Optical Filter Placement

Due to the unique optical layout of the C6, it is critical that any optional filters used with the Selectable Lasers Module are placed in the proper position for optimal performance.

WARNING: The 630/30 bandpass filter provided with the Selectable Lasers Kit should only be used when operating in the *4 blue* configuration. Using the 630/30 filter when operating in any other configuration may damage the corresponding detector due to unfiltered red laser signal.

Use the tables below as a guide to optical filter placement for various fluorochrome combinations:

Table E-1. *3 Blue 1 Red:* Configuration 1 (Standard Filters)

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC, GFP, CFSE
FL2	**585/40**	PE, PI PE-Texas Red
FL3	**670 LP**	PerCP-Cy5.5 PE-Cy5, PE-Cy7
FL4	**675/25**	APC Alexa-647 PE-Cy5

Table E-2. *3 Blue 1 Red:* Configuration 2

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC, GFP, CFSE
FL2	**585/40**	PE
FL3	**610/20**	PI PE-Texas Red
FL4	**675/25**	APC Alexa-647 PE-Cy5

NOTE: When operating in the *3 blue 1 red* configuration, place either the 610/20 bandpass or the 670 LP filters in position FL3. For best results when analyzing PE and PE-Texas Red (PE-TR) simultaneously, select the filters with the following signal-intensity considerations in mind:

- PE-bright, PE-TR-moderate to bright: FL3 = 670 LP
- PE-dim to moderate, PE-TR any level: FL3 = 610/20
- PE-bright, PE-TR dim: may be difficult to separate; consider using the *4 blue* configuration with a 630/30 in FL4 to detect PE-TR.

Table E-3. *2 Blue 2 Red* Configuration

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC, GFP, CFSE
FL2	**585/40**	PE, PI PE-Texas Red
FL3	**780/60**	APC-Cy7 (and equivalents)
FL4	**675/25**	APC (and equivalents)

Table E-4. *4 Blue:* Configuration 1

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC, GFP, CFSE
FL2	**585/40**	PE, PI PE-Texas Red
FL3	**780/60**	PE-Cy7 (and equivalents)
FL4	**675/25**	PerCP-Cy5.5 PE-Cy5

Table E-5. *4 Blue:* Configuration 2

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC, GFP, CFSE
FL2	**585/40**	PE
FL3	**780/60**	PE-Cy7 (and equivalents)
FL4	**610/20**	PI
	630/30	PE-Texas Red

Table E-6. *4 Blue:* Configuration 3

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC, GFP, CFSE
FL2	**585/40**	PE
FL3	**675/25**	PerCP-Cy5.5 PE-Cy5
FL4	**610/20**	PI
	630/30	PE-Texas Red

E.5 Selectable Laser Application Examples

E.5.1 *2 Blue 2 Red* Configuration Examples

Table E-7. Detector and Filter Configuration for *2 Blue 2 Red*, Examples 1 and 2

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC
FL2	**585/40**	PE
FL3	**780/60**	APC-Cy7
FL4	**675/25**	APC

Example 1: *2 Blue 2 Red* for Simultaneous FITC, PE, APC and APC-Cy7

The following images show the results for a 4-color analysis of $CD3^+CD4^+$ cells. Antibodies used were CD3-APC-Cy7 (UCHT1,BioLegend), CD4-APC (OKT4, eBioscience), CD45RA-FITC (HI100, eBioscience), and CD45RO-PE (UCHL, eBioscience).

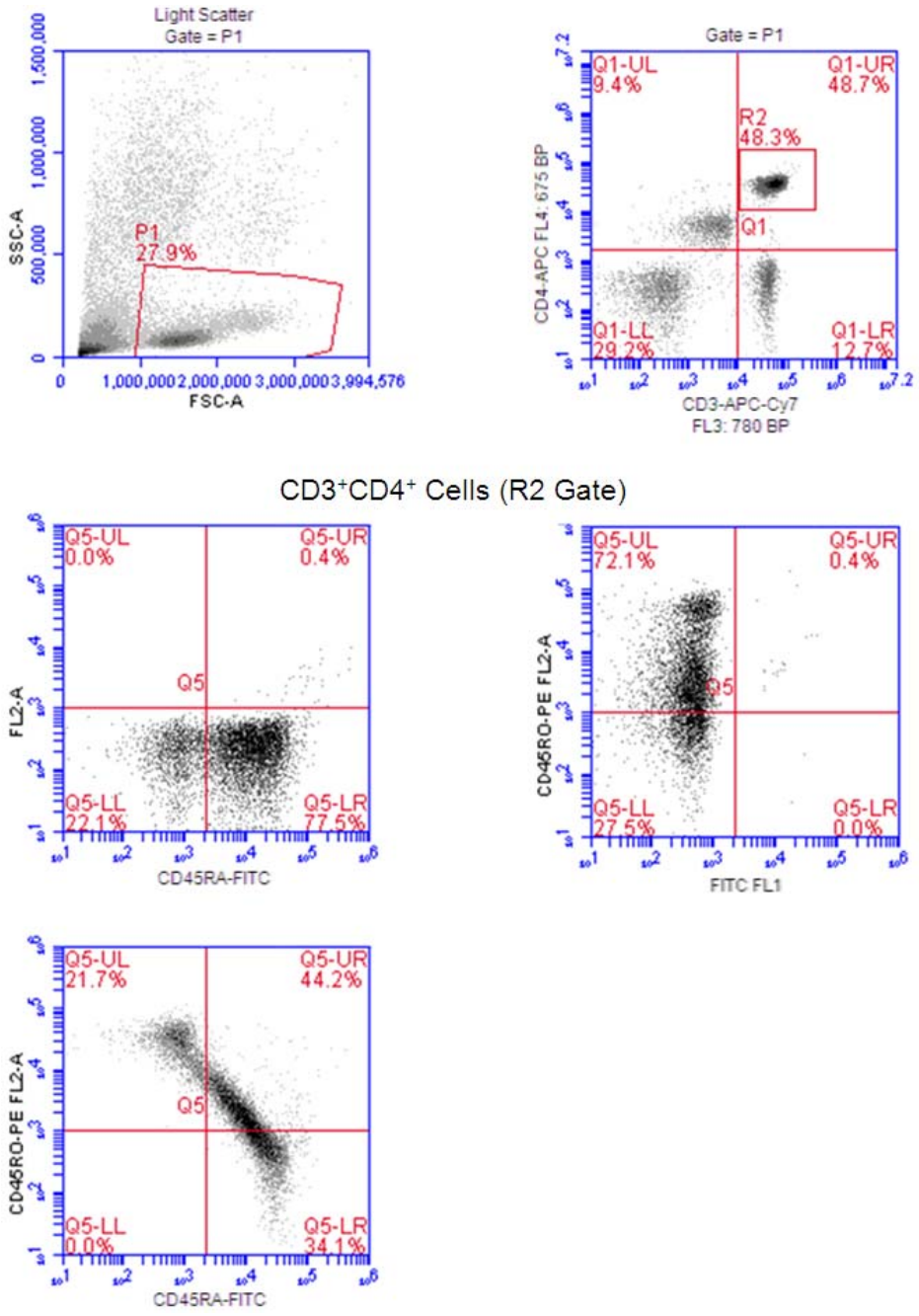
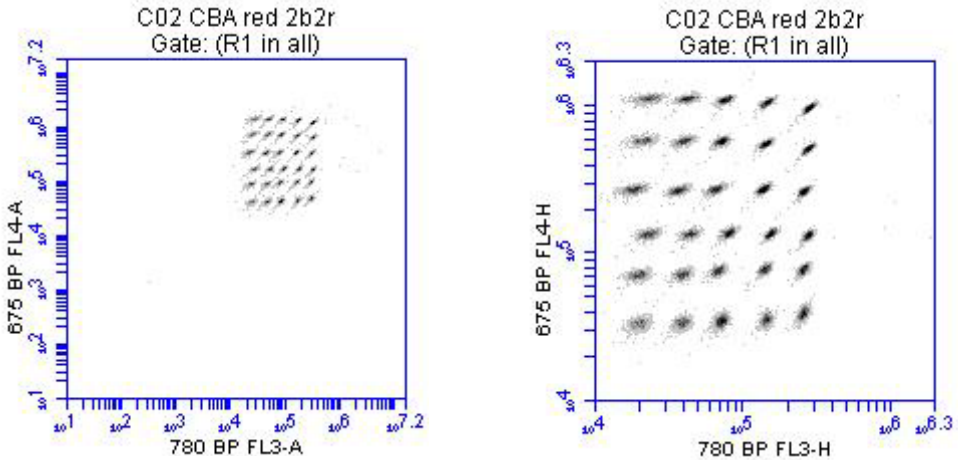


Figure E-12. Gating Example Using the *2 Blue 2 Red* Configuration

Example 2: *2 Blue 2 Red* with Cytokine Bead Standards Array (BD #51-9004679)

The following images show the results for a cytokine bead array.



Unzoomed View, Area

Zoomed View, Height

Figure E-13. Standard Beads (30-plex) for a Cytokine Bead Array

E.5.2 *4 Blue* Configuration Examples

Example 1: *4 Blue* FITC , PE, PE-Cy5, PE-Cy7: Human Peripheral Blood Subsets

Table E-8. Detector and Filter Configuration for Selectable Lasers *4 Blue* Example 1

Detector	*Filter*	*Fluorochrome*
FL1	530/30	FITC
FL2	585/40	PE
FL3	780/60	PE-Cy7
FL4	675/25	PE-Cy5

The following images show CD45-FITC vs. SSC gating of lymphocytes (zoomed in, left; zoomed out, right). HPB was stained with CD45-FITC (HI30, eBioscience), CD8-PE (OKT8, eBioscience), CD3-PE-Cy5 (UCHT1, Biolegend), and CD4-PE-Cy7 (RPA-T4, eBioscience).

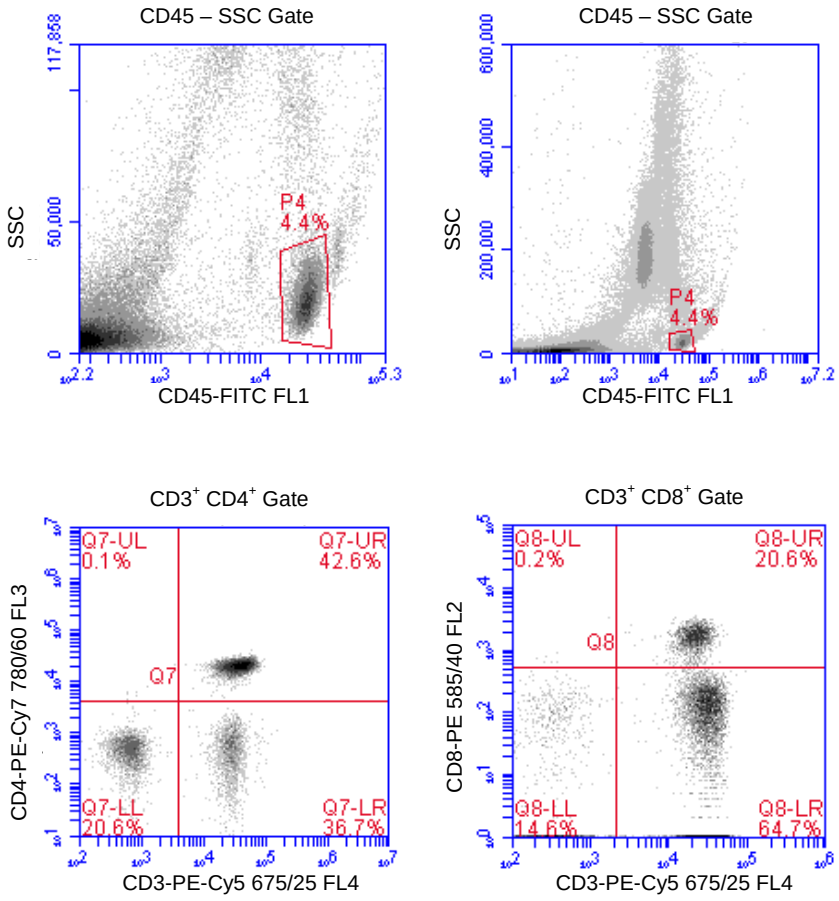


Figure E-14. *4 Blue* Configuration and the Optional 780/60 Optical Filter at Detector FL3

Example 2: *4 Blue* FITC, PE, PE-Cy5, PE-TxRed: Human Peripheral Blood Subsets

Table E-9. Detector and Filter Configuration for Selectable Lasers *4 Blue,* Example 2

Detector	*Filter*	*Fluorochrome*
FL1	**530/30**	FITC
FL2	**585/40**	PE
FL3	**675/25**	PE-Cy5
FL4	**630/30**	PE-Texas Red

The following images show the results for example 2. The color compensation value to correct the spillover of PE (FL2) into the PE-TexasRed (FL4) detector may be in the range of 70% to 90%.

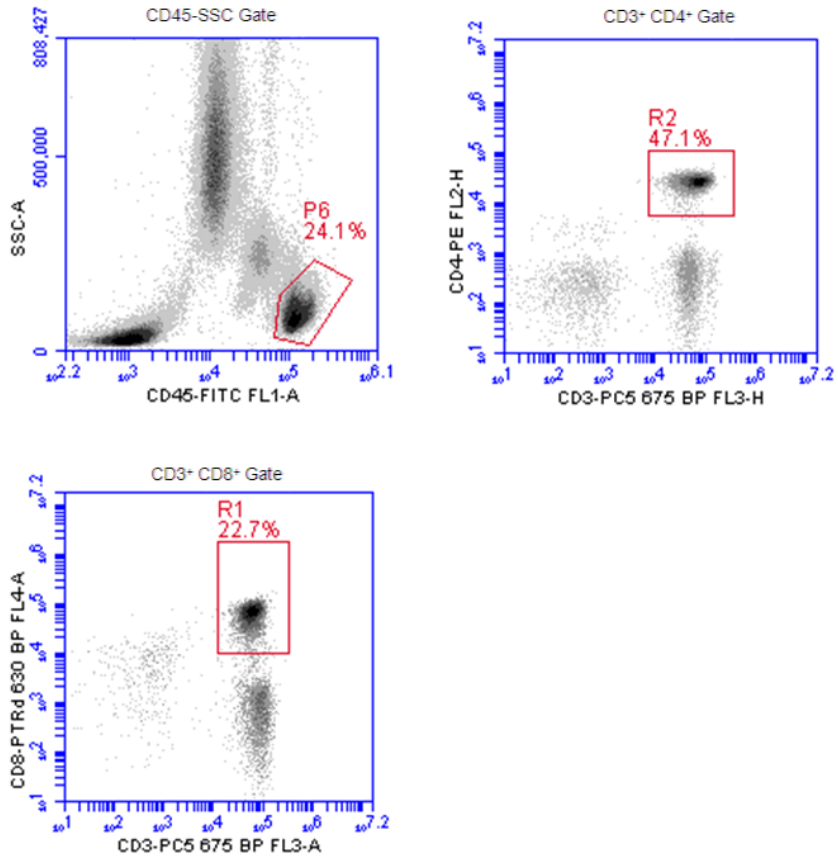


Figure E-15. HPB Stained with CD45-FITC, CD4-PE, CD8-PE-TexasRed and CD3-PE-Cy5

APPENDIX F FCS KEYWORDS

The following tables list all of the keywords CFlow uses in FCS files.

STANDARD FCS TAGS IN FCS FILES EXPORTED BY CFLOW OFTWARE

Table F-1. Standard FCS Tags

FCS Tag	*Description*	*CFlow*
$FIL	Filename including .fcs extension.	Save file dialog.
$SMNO	Sample name.	Sample name field above the Well Grid.
$DATATYPE	The data type of the actual values for each event. It is always “I” for unsigned binary integers.	Unchangeable.
$MODE	The mode of the data. It is always “L” for list mode where the data is in the order described by the $Pn keywords.	Unchangeable.
$BYTEORD	Order in which data bytes are written, least to most significant. It is always 4,3,2,1.	Unchangeable.
$NEXTDATA	The byte offset for an additional dataset in the file. Accuri files always specify 0 since the files only contain 1 dataset.	Unchangeable.
$PAR	Total number of parameters stored in the dataset. All datasets have 14 parameters.	Unchangeable.
$PnB	For parameter N, the number of bits for each binary value. The number is always 32 since integers are stored in 32 bits in Java.	Unchangeable.
$PnR	The range of parameter N. The range for all parameters is always 16777216.	Unchangeable.
$PnN	The name of parameter N. Parameters are the default values from CFlow.	Unchangeable.
$PnE	For parameter N, this denotes if linear or logarithmic amplifiers are used. It is always 0,0 because linear values for data are always saved. This is an optional tag.	Unchangeable.
$PnS	The name of the florescence stain or probe used for parameter N. This tag is used for the custom parameter name.	Rename parameter dialog.
$TOT	Number of objects stored in the data list. The cumulative event total for the sample.	Determined by the amount of events acquired by user.

FCS Tag	*Description*	*CFlow*
$DATE	The date the represented sample was last acquired into DD-MMM-YYYY.	Defined by the beginning time of the most recent acquisition. Set by the computer's clock.
$CYT	The name of the cytometer used for the measurement. It is always Accuri C6.	Unchangeable.
$SPILLOVER	The standard tag for color compensation.	Determined by values entered into Color Compensation dialog.
$TIMESTEP	Hard-coded value of 0.1, which is in seconds.	Unchangeable.
$PROJ	Represents the name of the workspace, which is also the name of the .c6 file (before the .c6 extension). If exporting an FCS file, this value is the name of the file before the .fcs extension.	Saving as a different file, importing an FCS file, or exporting an FCS file would change this value.
$BTIM	The beginning time of acquisition of the first event.	Unchangeable.
$ETIM	The end time of acquisition of the last event.	Unchangeable.
$VOL	Total volume in nanoliters.	Collecting more events would change the total volume.
$BEGINSTEXT	Default FCS 3.0 tag to mark the beginning of the text section.	Unchangeable.
$ENDSTEXT	Default FCS 3.0 tag to mark the end of the text section.	Unchangeable.
$BEGINANALYSIS	Default FCS 3.0 tag to mark the beginning of the analysis section.	Unchangeable.
$ENDANALYSIS	Default FCS 3.0 tag to mark the end of the analysis section.	Unchangeable.
$BEGINDATA	Default FCS 3.0 tag to mark the beginning of the data section.	Unchangeable.
$ENDDATA	Default FCS 3.0 tag to mark the end of the data section.	Unchangeable.

CUSTOM TAGS IN FCS FILES EXPORTED BY CFLOW

Table F-2. Custom FCS Tags

Tag	*Description*	*CFlow*
#CFLOWDECADESN	The number of decades for parameter N. It is always 7.224719870049579.	Unchangeable.
#CFLOW4COLORCOMP	A list of the percent values as entered into the color compensation window.	Determined by values entered into Color Compensation dialog.
#CFLOWCAPTUREDDATE	The date of the last time the represented sample was acquired into expressed in milliseconds since Jan 1, 1970.	Defined by the beginning time of the most recent acquisition. Set by the computer's clock.
PNVIRTUALGAIN	The VirtualGain set for parameter N, where 1.0 means no VirtualGain.	Set by user in the VirtualGain window.
#SAMPLE	Value is either the well code or the sample rename (if one exists).	Can be changed by renaming the sample in the application. If no rename exists, importing an FCS file into a different well than the one collected in will change this value.
#ATIM	Cumulative acquisition time.	Can be changed by acquiring more data.
#SPACERS	Used to pad the text, analysis, and data sections of the FCS file.	Unchangeable.

FCS 3.0 DEFINED TAGS NOT IN CFLOW EXPORTED FCS FILES

Table F-3. FCS Tags Not in CFlow Exported Files

Tag	*Description*
$COMP	Amount of fluorescence compensation employed during collection. This replaces the $DFCiTOj tag from FCS 2.0.
$TIMESTEP	Absolute measure of time used in kinetic analysis.
$UNICODE	Enables usage of certain keywords in non-English languages. This is optional.
$PnE	For parameter N, this denotes if linear or logarithmic amplifiers are used. CFlow always uses 0,0 because CFlow always saves linear values for the data. Mandatory in 3.0.
$PnN	When time is collected, the keyword value of the time-parameter name must now be the string "TIME".
CRC	An optional 16-bit Cyclic Redundancy Check has been added to the end of each dataset.

APPENDIX G EXAMPLE .CSV FILE

The following table is an exported bead log file in .csv format.

Table G-1. Example .csv File

FSC-A	SSC-A	FL1-A	FL2-A	FL3-A	FL4-A	FSC-H	SSC-H	FL1-H	FL2-H	FL3-H	FL4-H	Width	Time
118075	95988	456	294	272	851	607048	422139	1473	815	714	2875	43	121
117720	88712	576	268	283	881	601519	374399	1417	719	533	2521	43	121
110042	85241	2866	1872	2126	7954	583439	372881	7325	5042	5356	28644	41	121
114296	67129	9034	5419	6515	24375	583940	287922	23759	14273	18009	91638	42	121
118532	101077	56704	38096	37950	73004	591859	437256	147685	95718	89739	233762	43	121
114148	89976	1035	714	496	2868	586298	383138	2897	1998	2454	10256	42	121
116901	77515	136085	104961	155282	133610	581299	331039	344734	272212	394709	440201	42	121
111757	80760	16897	10281	11171	39681	580904	354623	45060	26759	29697	149886	43	122
113937	86275	1193	763	921	2540	580677	380168	2632	1748	2965	8335	42	122
112549	105640	125303	101393	154812	137072	563859	450332	313047	252427	393460	470855	43	122
112085	71263	1029	759	227	2902	567995	316559	2683	1915	1352	10755	41	122
118052	96038	95	72	0	129	592987	405663	384	121	599	799	43	122
110943	89761	133064	100253	161526	143719	576133	385371	355359	266257	414414	478612	42	122
115882	84264	1047	593	984	3045	573668	347709	3082	1507	2678	9212	42	122
109415	85656	1315	671	523	2890	583643	380640	3530	1980	2950	10823	41	122
112818	87317	0	0	0	0	583281	385421	317	146	397	463	42	122
116118	87517	8382	5371	5645	22063	575042	362503	20362	14123	11901	73736	43	122
122745	100658	8036	5433	5483	30389	612061	426570	20973	13186	14029	100238	45	122
117550	94310	422	107	140	695	594473	403387	1165	492	604	2492	42	122
107921	90101	1198	647	654	2808	572735	398355	3718	1765	1884	9203	41	122
110195	111820	21375	12491	12746	72825	546675	464189	55839	32764	32063	216207	43	122
115341	93977	16101	10748	11758	37634	588991	393280	41618	27052	29625	133265	44	122
110606	73288	8681	4925	5365	21686	572786	320110	24384	13815	12568	77871	42	122
112173	66739	129974	96461	151427	124808	574229	291566	354745	262399	416530	469631	42	122

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